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Chapter 13
Prevention and Treatment of Botulism

Michael Adler, Nizamettin Gul, Edward Eitzen, George Oyler and Brian Molles

Abstract Concerns regarding botulinum neurotoxins (BoNTs) as biowarfare and bioterrorist agents have generated interest in developing medical countermeasures for protection against the neurotoxins. Efforts have focused on improvements in the available vaccines and antisera and de novo discovery of pharmacological inhibitors of toxin action. This chapter reviews the various approaches taken to develop next-generation vaccines, antitoxins and pharmacological treatments against intoxication by the BoNTs. The historical progression, current status and future trends are described.

Keywords Antitoxin · Bioterrorism · Botulinum neurotoxin · Botulism · Metalloprotease inhibitors · Vaccine

13.1 Introduction

13.1.1 Background

The botulinum neurotoxins (BoNTs) are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality [26, 73, 104, 113, 146, 154, 162, 196]. The toxicity of the BoNTs stems from their potent and selective inhibition of acetylcholine (ACh) release at the neuromuscular junction, autonomic ganglia and structures innervated by the parasympathetic branch

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of the autonomic nervous system [19, 44, 46, 83, 86, 126, 159]. Paradoxically, this selective inhibition of ACh release has also enabled BoNT/A to become a highly useful therapeutic agent [22, 41, 43, 59, 140].

Since its approval in 1989 as an orphan drug for the treatment of strabismus, hemifacial spasm and blepharospasm, BoNT/A has come to be regarded as the treatment of choice for an increasing number of neurologic, autonomic and cosmetic conditions [43, 55, 82, 85, 222, 264]. The ability of BoNT to serve in this role is based on its exquisite selectivity for cholinergic nerve terminals, its long duration of action and its ability to remain localized near the intended target when injected at low concentrations and volumes [139, 153].

Although we have learned to harness the therapeutic benefits of BoNT in ways that were not even imagined when the neurotoxin was first approved as an orphan drug, we should not lose sight of the fact that BoNT is inherently a highly lethal toxin, that outbreaks of botulism with devastating consequences continue to occur [73, 76, 198, 277] (Chap. 12 of this volume) and that recovery from botulism can require months of intensive care and rehabilitation, often leaving patients with long-lasting physical and psychological trauma [68, 69, 132, 150, 151, 266]. After severe intoxication by BoNT, restoration of normal muscle function, exercise tolerance and cardiovascular fitness have been reported to take nearly a year for BoNT/F [239], more than 2 years for BoNT/B [266], and greater than 5 years for BoNT/A [151].

13.1.2 BoNT as a Bioterrorist Weapon

In addition to natural outbreaks, the potential use of BoNT by hostile nations or terrorist groups has been a growing concern [26, 29, 101, 116, 162, 191, 196, 198, 219, 263]. The ability of BoNT to cause mass casualties has led to its designation as a Tier 1 select toxin by the US Department of Health and Human Services (HHS), the only noninfectious agent to receive this designation (http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). The threat of battlefield deployment of BoNT has diminished somewhat following the dissolution of the Soviet Union in 1991 and the regime change in Iraq in 2003. However, use by terrorists has become an increasing concern due to widespread availability of BoNT from both legitimate and illegitimate sources, coupled with ease of concealment and inherent vulnerabilities of our modern food and beverage distribution systems [265]. These factors, in conjunction with a rise of religious fundamentalism and proliferation of failed nations and those that support or sponsor terrorism, make an attack by BoNT more probable than in previous generations [21, 191, 196].

A bioterrorist attack with BoNT is likely to involve aerosol delivery or deliberate contamination of food, beverage or animal feed [1, 21, 26, 162, 263]. The pattern of botulism following a terrorist attack would be expected to resemble that observed after a natural outbreak, except the former may involve a larger number of casualties, originate at multiple locations and, in the case of aerosol exposure, lack an

easily identifiable source [1, 26, 101]. As with naturally occurring outbreaks, signs and symptoms following a terrorist strike by BoNT would consist of cranial nerve palsies, followed by symmetrical descending muscle weakness and respiratory collapse [73, 191, 196, 238]. BoNTs are less potent by inhalation than by injection and least potent by ingestion [26, 162]. The lower potency by the latter routes may be related to the need for BoNT to undergo transcytosis across airway or intestinal epithelial cells prior to entering the general circulation, whereas injection provides direct access to the bloodstream [18]. Moreover, ingested toxin must also overcome the hostile environment of the gastrointestinal (GI) tract (low pH and proteolytic enzymes), a process which is aided by association with a specific nontoxic non-hemagglutinin accessory protein. This protein is co-secreted by *Clostridium botulinum* and is able to shield the neurotoxin by providing it with complementary binding surfaces [107].

13.1.3 Medical Management of BoNT Intoxication

Treatment options for BoNT intoxication have changed little in principle over the past 40 years, although the therapies, guidelines and doctrines have undergone periodic refinement. Medical countermeasures consist of timely administration of antitoxin for those individuals exhibiting clear signs of exposure and treatment in an intensive care facility until patients can be discharged to lower levels of care such as to a rehabilitation unit [196]. Patients exhibiting respiratory collapse would also require mechanical ventilation in addition to the above measures, sometimes for extensive periods [34, 65, 238, 249].

In addition to these measures, vaccination with pentavalent botulinum toxoid (PBT) vaccine was recommended, until recently, for individuals in high-risk groups. Since development of active immunity to BoNT is relatively slow, vaccination would have to be initiated well before an outbreak and would be of no benefit after an exposure to BoNT [94, 202, 221]. On 30 November 2011, PBT was withdrawn by the US Centers for Disease Control and Prevention (CDC) due to problems with reactogenicity and declining immunogenicity; new recombinant vaccines are under development but not yet licensed by the US Food and Drug Administration (FDA) (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6042a3.htm?s_cid=;mm6042a3_x). BoNT vaccines are discussed in detail in Sect. 13.2.1.

It is generally agreed that a large-scale bioterrorist attack would overwhelm our intensive care facilities, and vaccination is not considered to be practical for civilians since vaccinated individuals would be precluded from benefiting from the medical uses of BoNT [191, 202]. The indications for the latter have expanded markedly from rare focal dystonias to more common medical conditions such as genitourinary disorders, hyperhidrosis, pain, headache and neuropathy [222, 264]. Candidates for BoNT therapy now encompass a sizable fraction of the population [41, 43, 82]; consequently, when improved vaccines become available, vaccination should only be considered after a careful risk–benefit analysis [230]. In addition,

reengineering of toxins has opened up additional therapeutic opportunities for treatment of conditions such as chronic pain [98, 157] and asthma [99]. These reengineered toxins are likely to be ineffective in BoNT-immunized individuals [84].

Since it is expected that most victims of a bioterrorist attack with BoNT would not be vaccinated, symptomatic treatment would need to be supplemented by infusion of antitoxin to prevent continued internalization of BoNT into target tissues. The current product is a despeciated heptavalent botulism antitoxin (HBAT) developed originally by the US Army Medical Research Institute of Infectious Diseases (USAMRIID) in conjunction with the University of Minnesota [119]. As with earlier antitoxins, HBAT is effective in reducing the severity of BoNT intoxication, as long as it is administered while active toxin is still circulating in the bloodstream [154].

To overcome this time constraint for therapy and to accelerate recovery, specific pharmacological agents to counteract BoNT intoxication would be desirable, either as stand-alone treatments or as adjuncts to antitoxin [7, 244]. The purpose of this chapter is to use the insights gained in our understanding of the mechanism of BoNT action, especially during the past two decades, to establish a conceptual framework within which to develop effective treatment strategies for intoxication, and to evaluate current and emerging treatment options. Important advances have been made in a number of critical areas. These include identification of antitoxin-binding epitopes [28, 32], determination of BoNT pharmacokinetics in animal models [61, 193], identification of the protein receptors for cell surface binding [79, 80, 123, 173], a more precise characterization of the translocation channels [94] and resolution of the crystal structure of BoNT and its functional domains [14, 42, 138, 247, 248]. Many of these discoveries were set in motion following recognition of the zinc metalloprotease action of the BoNT light chain (LC) [169], and subsequent identification of their SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein targets [168, 204, 205, 206]. The SNARE proteins targeted by BoNT, namely SNAP-25, synaptobrevin and syntaxin, are intimately involved in synaptic vesicle exocytosis, and their cleavage by BoNT is the key event that precedes the onset of toxin-mediated paralysis [182]. Continued advances in our understanding of the mechanism of BoNT-mediated intoxication are expected to lead to sustained improvements in our treatment options in the years ahead.

13.2 Vaccines

13.2.1 Botulinum Pentavalent Toxoid Vaccine

Other than physical protection, vaccination is the sole means to prevent botulism. From the earliest stages of vaccine development, it was recognized that a separate toxoid would be required to generate protective antibodies for each serotype, since neutralizing BoNT antibodies exhibited little cross-reactivity with non-homologous

BoNT serotypes [221]. In fact, the lack of cross-reactivity was the basis for designation of the eight distinct serotypes of BoNT [32, 114]. For the past half century, prophylaxis against botulism had been carried out by use of PBT vaccine developed by the US Army at Fort Detrick, Maryland [94]. The pentavalent formulation encompassed all of the serotypes (A, B, C1, D, E) that were known at the time of original production in 1958 and included the three serotypes that were responsible for most human outbreaks (A, B, E) [94, 114, 221]. PBT is generally administered to personnel at high risk of exposure such as those employed in botulinum research laboratories, BoNT production facilities and public health laboratories that investigate botulism outbreaks or military personnel deployed to regions with high potential for bioterrorism or biological warfare [73, 202, 221]. The individual monovalent toxoid components of PBT were produced separately in bulk culture, partially purified, inactivated with formalin (0.022%) and adsorbed on aluminum phosphate (adjuvant) to increase immunogenicity [94]. PBT vaccine was generated by blending the five monovalent toxoids, which were preserved with 0.01% thimerosal and bottled in multiuse vials [23, 221]. Vaccination of human volunteers with a series of three deep subcutaneous injections of PBT over a 10- to 12-week period followed by a booster at 52 weeks led to measurable titers in virtually all vaccinees.

There is compelling evidence for the efficacy of PBT from animal studies, from which human efficacy can be inferred. Thus, guinea pigs vaccinated with PBT were able to survive a challenge by up to 10^6 mouse median lethal dose (MLD_{50}) of BoNT [94], and passive transfer of antibodies from individuals vaccinated with PBT protected guinea pigs [103] and nonhuman primates (NHPs) from aerosol challenge by lethal doses of BoNT [100]. In addition, purified human immunoglobulins from volunteers vaccinated with PBT have been found to be highly effective in the treatment of infant botulism ([27], cf. Sect. 13.3.4).

PBT vaccine has been available from the CDC as an investigational new drug (IND) since 1965 for civilians at risk (IND 161) and subsequently for deploying military personnel (IND 3723, US Army Office of the Surgeon General). From 1965 to the present time, more than 20,000 injections have been given to those in the high-risk category, and more than 8,000 injections were administered to military personnel. In spite of its long history, PBT has never been licensed by the FDA. This is largely because the FDA's Animal Rule, which allows for licensure of vaccines (and approval of drugs) in the absence of human efficacy data, only came into effect in July 2002, well after PBT was developed [113].

Since 1979, five separate lots of PBT were produced, all using the same monovalent toxoids. New lots were necessitated by shortages in supply or reductions in potency of some of the individual serotypes. The most recent lot (PBP-003) has been available since 1994 [202]. Due largely to the high levels of formalin (to prevent reactivation of toxoid to toxin) and thimerosal in the final product, PBT is reactogenic, although most adverse events have been reported to be local rather than systemic: pain and erythema/induration at the injection site. For lot PBP-003, moderate local reactions (erythema/induration between 30 mm and 120 mm) were associated with 12% of vaccinations, and severe local reactions (erythema/induration more than 120 mm, or axillary lymph node enlargement and/or tenderness) with 2%

of vaccinations. Systemic adverse events (fever, malaise, headache, myalgia) were mild and occurred in 7% of vaccinees. Reactions were more frequent after boosters than after the primary series [202].

13.2.2 Limitations of PBT Vaccine

In spite of its long use, the PBT vaccine was found to have a number of drawbacks, which has led to the search for new-generation vaccines for BoNT intoxication. Among these are high reactogenicity, poor immunogenicity and absence of toxoids for BoNT/F or BoNT/G, both of which were discovered considerably later than the first five serotypes [105, 166]. Although BoNT/F and BoNT/G have been implicated in only a small number of human intoxications [147, 241], both are considered to be potential agents of bioterrorism. With regard to immunogenicity, achieving optimal protective titers requires injections at 0, 2 and 12 weeks (primary series) plus a boost at 52 weeks; annual boosters were recommended to maintain titers [236]. Antibody titers were observed to fall significantly between the end of the primary series and the first annual booster, creating a long window of vulnerability [221]. This problem was addressed in 2004 by adding a 6-month booster.

There have also been periodic issues with reductions in potency, especially for serotypes B and E [202]. The low immunogenicity and loss of potency were presumably the result of the age and relative impurity of the individual toxoids. The monovalent components were manufactured between 1969 and 1971 and contained only 10–15% protein [202]. These problems could be alleviated, in principle, by formulation of toxoids with higher purity [127]. However, the current safety and surety concerns over the large-scale toxin production that would be needed to support the vaccine effort and the increased regulatory requirement by the FDA for product safety make this approach impractical.

13.2.3 Discontinuation of PBT

As of November 30, 2011, the CDC terminated release of PBT for individuals at high risk of occupational exposure to BoNT [57]. This decision was based on the reduction in immunogenicity coupled with a progressive rise in reactogenicity of PBT, as described earlier. To allow current vaccinees to complete the primary series, PBT remained available until May 31, 2012. However, no new personnel were permitted to be vaccinated with PBT, and no licensed or IND vaccine for botulism is currently available in the USA to replace PBT [57].

It has been recognized for more than 20 years that new vaccine candidates could overcome many of the problems associated with the traditional vaccine, and alternative approaches to develop vaccines against the BoNTs were initiated in the early 1990s for eventual replacement of PBT [67]. Many of these efforts are still being pursued, including recombinant subunit vaccines based on the toxin-binding

domain, the combined catalytic and translocation domains, or holotoxin rendered inactive by mutations in the catalytic domain. These will be discussed in the following sections

13.2.4 Recombinant Botulinum Vaccines

Improvements in recombinant DNA techniques allowed for elucidation of the nucleotide and deduced amino acid sequence of clostridial neurotoxins nearly three decades ago [87, 130, 252, 265]. These advances enabled expression of nontoxic fragments of BoNT for use as vaccine candidates. Unlike toxoids, recombinant antigens could be produced in quantities sufficient for vaccine development without the need for large-scale culture of *C. botulinum* and its associated surety and biosafety risks, high cost, and need for chemical detoxification.

13.2.4.1 Recombinant Subunit Vaccines

The first demonstration that a nontoxic component of BoNT/A was able to generate protective antibodies was provided by investigators at USAMRIID [67]. These authors constructed a synthetic gene coding for the ~ 50-kDa binding domain of BoNT/A (Hc) which, unlike the native clostridial gene, could be readily expressed in *Escherichia coli*. The recombinant Hc, corresponding to the C-terminal half of the heavy chain (HC), was selected since it is the most antigenic component of BoNT and the one most likely to produce neutralizing antibodies [28, 33, 254]. An additional advantage of Hc is that it is nontoxic since it lacks both the translocation domain (H_N) and the catalytically active LC [229, 233]. After purification and adsorption on aluminum hydroxide adjuvant, mice were immunized with Hc. A series of three vaccinations at 0, 2 and 4 weeks protected mice against a 10^6-MLD_{50} challenge dose of BoNT/A at week 5. This pioneering study was inspired by earlier efforts with the related tetanus neurotoxin (TeNT) [92], and it clearly demonstrated the feasibility of using a nontoxic fragment to protect against botulism.

Since BoNT/A Hc appeared to be a promising vaccine candidate capable of replacing PBT, Smith and coworkers modified the synthetic Hc gene for expression in the yeast *Pichia pastoris* to take advantage of the superior attributes of this system (high yields, lack of endotoxin and ease of purification), and subsequently extended the recombinant vaccine effort to seven BoNT serotypes [52, 53, 124, 189, 235, 260, 261]. In preclinical studies, recombinant BoNT/A Hc was found to be protective in mice, and Hc of serotypes A and B were also shown to be protective in NHPs challenged by aerosol exposure [40, 113]. Moreover, neutralizing antibody titers were detected for up to 2 years in NHPs following vaccination with Hc domains [40].

To prepare for replacement of PBT by a recombinant vaccine, the US Chemical Biological Medical Systems-Joint Vaccine Acquisition Program (CBMS-JVAP) has been working with DynPort Vaccine Company LLC (DVC) to transition a bivalent

serotype A and B Hc recombinant vaccine (rBV A/B) for licensure under the FDA's Animal Rule. The vaccine was developed against BoNT/A subtype A1 and BoNT/B subtype B1 [113].

In 2004, DVC submitted an IND application to the FDA to carry out phase 1 clinical trials on this vaccine in a group of healthy adult volunteers [236]. The bivalent vaccine was well tolerated in the study population and stimulated serotype-specific neutralizing antibodies at all dosage levels tested. Moreover, passive transfer of antibodies from human subjects to guinea pigs was protective when animals were challenged with a 10-MLD_{50} dose of BoNT/A or BoNT/B [202].

The rBV A/B program was granted Fast Track designation by the FDA, and phase 2 trials were begun in 2008. The trials involved a study population of 440 healthy adult volunteers and assessed the safety of rBV A/B over an 18-month period. Vaccine or placebo was administered at two dosing schedules: 0, 28 and 182 days or 0, 56 and 182 days. Antibody levels were then measured at prescribed time intervals. This study was successfully completed in February 2011. Licensure must now await completion of phase 3 studies. If successful in phase 3, rBV A/B is likely to be the first vaccine approved under the FDA's Animal Rule (http://assets1.csc.com/dvc/downloads/DVC_Botulinum_Vaccine_Case_Study_May_2010.pdf).

Even if rBV A/B achieves licensure as planned, it still leaves the remaining serotypes of BoNT without an approved vaccine. In limited preclinical studies, recombinant Hc fragments of serotypes C and D were also found to be effective vaccine candidates [261], both alone and in combination. Further, a heptavalent Hc-derived vaccine was found to protect mice challenged by 10,000 intraperitoneal (i.p.) MLD_{50} units of each BoNT serotype [31]. At this time, however, there are no plans to conduct advanced studies on these serotypes, largely due to resource limitations.

13.2.4.2 Other Recombinant Vaccine Candidates

In addition to Hc-derived vaccines, a number of other recombinant products have been studied at the preclinical level that may be candidates for future vaccine development. Among these are subunit vaccines consisting of the combined catalytic and translocation domains (LCH_N) [220], subunit vaccines coupled to viral vectors for enhanced immunogenicity [141, 171, 275] and catalytically inactive holotoxin that contains mutations in the LC, rendering the molecule nontoxic (holotoxoid) [186, 262]. This holotoxoid would appear to be the ideal immunogen, since it could elicit neutralizing antibodies to the binding, translocation and catalytic domains of BoNT. Moreover, the holotoxoid could also offer protection following challenge with a reengineered toxin in which one or more domains were altered to evade subunit vaccines targeting a single domain.

Both a double LC mutant of BoNT/A expressed in *E. coli* (R362A, Y365F) [186] and a triple LC mutant expressed in *P. pastoris* (H223A, E224A, H227A) [262] were found to be highly protective in mice. The triple mutant was in fact shown to protect against three of the five subtypes of BoNT/A (A1, A2, A3) after a single

injection, whereas the Hc-derived vaccine was only effective against challenge by A1 under this condition [262]. Although these differences were less pronounced following multiple vaccinations, the atoxic mutant holotoxoid still has the advantage of eliciting protection with less delay, which is significant for enabling rapid military deployment to regions with potential BoNT exposure.

Subtypes within serotypes arise from variations in the primary structure of the toxins, and have significant impact on protective strategies since vaccines and antitoxins are generally less effective in protecting against dissimilar BoNT subtypes [192, 220, 237, 262].

13.2.5 Mucosal Vaccine Delivery

Vaccines delivered parenterally produce antibodies that can neutralize BoNT only after it has gained access to the bloodstream. Vaccines delivered mucosally, on the other hand, are capable of inducing both systemic and mucosal immunity [66, 102, 183]. The addition of mucosal immunity is of potential advantage since botulism, other than wound or iatrogenic, involves initial binding to the airway or intestinal mucosa [133, 149, 194]. Xu et al. [270] demonstrated that intranasal (i.n.) delivery of a vaccine constructed from a replication-incompetent adenoviral vector encoding the Hc component of BoNT/C was able to elicit high levels of immunoglobulin A (IgA) in mucosal secretions and immunoglobulin G (IgG) in sera of mice 2 weeks after a single vaccination with 2×10^7 plaque-forming units. In addition, this dose protected 100% of mice against a 100-MLD_{50} challenge of BoNT/C at 7–27 weeks after vaccination. The rapid onset, persistence and ability to achieve protection after a single vaccination are remarkable and exceed the performance of traditional and other new-generation vaccine candidates. It is not clear, however, what role mucosal IgA played in the observed protection, since similar results were obtained when the adenoviral vaccine was injected intramuscularly [275]. Even if the mucosal protection was not the major factor in the findings of Xu et al. [270], i.n. delivery still has the advantage of increased efficiency and lower cost [194].

Based on the preceding, we have transitioned from having a single pentavalent toxoid vaccine for botulism (PBT), which was limited by low immunogenicity and high reactogenicity, and are in the process of developing a safer and more effective bivalent subunit vaccine (rBV A/B) that, in principle, can be extended to all serotypes [31]. There are other, perhaps even more promising candidates, such as atoxic holotoxins, that may provide protection against some deliberately modified forms of BoNT whose binding domain might not be neutralized by rBV A/B [186, 262]. Which, if any, additional vaccine candidates will be selected for development is yet unclear. In addition, the benefits of vaccination must be weighed against the considerable cost of developing new vaccines, the likelihood that BoNT intoxication can be successfully treated by a combination of antitoxin and a yet to be developed therapeutic and the probable loss of clinical benefit to the vaccinees.

13.3 Antitoxins

13.3.1 Rationale for Antitoxin Treatment

Although in use for more than four decades, equine antitoxins are still the only postexposure products available for limiting the severity of BoNT intoxication [73, 154]. The efficacy of antitoxins stems from their ability to neutralize BoNT in circulation and thus to prevent further internalization of toxin. Achieving this requires antitoxins to be administered early during the course of illness [115, 215, 249]. The temporal limitation of antitoxin treatment has long been appreciated [117] and is related to the fact that clostridial neurotoxins exert their actions inside the nerve terminal, where they are not susceptible to antibody neutralization [229]. Accordingly, at the time when signs and symptoms of botulism become apparent, a substantial quantity of toxin has already become internalized, and only the fraction that is still in the circulation is available to be neutralized.

In light of this limited therapeutic window, the question arises as to why are antitoxins not administered prophylactically, especially if the threat of BoNT exposure is imminent. There are two compelling reasons why antitoxins are not used without evidence of exposure; both are related to the equine origin of the product. First, equine-derived antitoxins have a high risk of hypersensitivity. The previously licensed trivalent ABE antitoxin (Table 13.1) was associated with a 9% incidence of hypersensitivity [35]. Of 268 patients studied retrospectively over an 11-year period, anaphylaxis was observed in nearly 2% of the study population within 10 min of antitoxin treatment, and nearly 4% in this group developed serum sickness 6–20 days after receiving antitoxin [35]. Second, equine antitoxins are expensive and difficult to produce. Until a substantial quantity of HBAT was delivered to the Strategic National Stockpile (SNS), BoNT antitoxins were only available in extremely limited supply, reflecting production difficulties, high cost, limitations of shelf life and the fact that naturally occurring botulism is a rare disease [215].

13.3.2 Efficacy of Antitoxin

Although equine-derived antitoxins have been demonstrated to be highly protective in animal studies [100], their efficacy in humans has never been established directly in double-blind placebo-controlled clinical trials. Instead, efficacy was inferred from retrospective studies on BoNT-intoxicated patients. In one such study involving 134 patients, those who received antitoxin within 24 h of onset of signs and symptoms had a lower fatality rate (10%) than those who received antitoxin after 24 h of onset (15%) or those who did not receive antitoxin (46%). In addition, patients who received antitoxin within 24 h had shorter hospital stays and spent fewer days on a ventilator than those who received antitoxin after 24 h [249]. To determine the time window for antitoxin administration more rigorously, it would be desirable to have data on plasma levels of BoNT in humans as a function of time

Table 13.1 Botulinum antitoxins

Product	Source	Years used	Availability	Status: year
Heptavalent botulism antitoxin (A–G) (HBAT)	Equine Fab, $F(ab')_2$	2008–current[a]	CDC	IND: 2010 Licensed: 2013
Bivalent A, B	Equine IgG	1999–2010	Withdrawn 2010	IND: 1999–2005; licensed: 2005–2010
Monovalent E	Equine IgG	2000–2010	Withdrawn 2010	IND: 1999
Trivalent A, B, E	Equine IgG	1960–1999	Withdrawn 1997[b]	Licensed: 1960
BIG-IV	Human IgG	2003–current	California Department of Health Services	Licensed in 2003 for infant botulism (BabyBIG®)

CDC US centers for disease control and prevention, *IND* investigational new drug, *IgG* immunoglobulin G, *BIG-IV* botulism immune globulin intravenous (Human)

[a] Until 2008, heptavalent botulism antitoxin (HBAT) was provided from US Army Medical Research Institute of Infectious Diseases (USAMRIID) to the US Centers for Disease Control and Prevention (CDC) on a compassionate basis for type F outbreaks since only types A, B, and E antitoxins were available from the CDC

[b] From 1997 to 1999, trivalent A, B, E was used only for type E outbreaks. Information on some of the dates for usage was provided by Dr. Susan Maslanka, Division of Foodborne, Bacterial, and Mycotic Diseases, National Center for Zoonotic, Vector-borne, and Enteric Diseases, CDC, Atlanta, GA, USA

following potential routes of exposure (ingestion, inhalation, wound, injection). Unfortunately, human pharmacokinetic data for BoNT are lacking since patients are often not seen by medical personnel until well after exposure, and, in addition, BoNT is so potent that the toxin is often difficult to detect by conventional methods, such as the mouse bioassay [267].

In a study conducted on patients with foodborne botulism, toxin was detected by the mouse bioassay in less than half of serum and stool samples examined within 3 days of ingestion (40–44 %) and in only 15–23 % of samples obtained outside of this time frame [269]. Ravichandran et al. [193] investigated the pharmacokinetics of BoNT/A in the mouse and rat by use of relatively high doses of ^{125}I-BoNT/A. The half-life of ^{125}I-BoNT/A in serum was found to be approximately 4 h in both species. As expected, when antitoxin was mixed with toxin and injected in animals, no deaths or signs of botulism were observed. However, when antitoxin was administered 10 min after BoNT, the former only prolonged survival but could not prevent death; if antitoxin was administered 20 min after BoNT, it was unable to even delay death. Similar results were obtained by Cheng et al. [61], using an electrochemiluminescence enzyme-linked immunosorbent assay for detection of BoNT. For lower doses of BoNT, such as those encountered during natural outbreaks, the treatment window is much longer, and antitoxin would confer some benefit as long as toxin remains in circulation [249].

Although it is commonly believed that circulating BoNT is cleared from the bloodstream within 1 or 2 days of exposure, clearance from blood may in fact take considerably longer after severe intoxication. The presence of BoNT in serum 3 days after clear manifestation of symptoms has been documented following an

outbreak of foodborne botulism [115]. Moreover, in the November 2004 Oakland Park, Florida outbreak, BoNT/A in excess of 40 times the estimated human median lethal dose was observed in the serum of one patient 4 days after receiving a massive overdose of toxin. The intoxication occurred during a cosmetic procedure in which the individual was injected for glabellar line reduction with research-grade BoNT/A that was incorrectly diluted [65, 243]. For such severely intoxicated patients, antitoxin administration may still be effective in limiting the severity and duration of illness, since it would prevent continued internalization of circulating toxin. In this context, it is of interest that blood components do not appear to bind, degrade, or alter BoNT, so toxin detected in blood can be considered to be active even days after intoxication [193]. From patient records examined between 1973 and 1980, Tacket et al. [249] concluded that the upper end for antitoxin efficacy was unknown. Unfortunately, we still do not have reliable data to establish this limit.

13.3.3 Equine Heptavalent Botulinum Antitoxin

Presently, the only antitoxin available in the USA for noninfant botulism is an equine HBAT manufactured by the Cangene Corporation of Canada for the CDC's Quarantine stations and for HHS's SNS. HBAT has replaced the licensed equine bivalent AB antitoxin (BAT-AB) originally made by Connaught Corporation of Canada and acquired by Aventis/Sanofi-Pasteur, and the investigational monovalent type E antitoxin (BAT-E) (Sanofi Pasteur). BAT-AB and BAT-E expired on March 12, 2010 [56].

HBAT is prepared from plasma of horses immunized with one of seven serotypes of BoNT toxoid and toxin. For each antitoxin serotype (A–G), purified $F(ab')_2$/Fab immunoglobulin fragments are produced by pepsin digestion of the IgG monomer. The Fc fragments are removed to minimize the risk of hypersensitivity reactions (despeciation). The final product contains $<2\%$ intact IgG and $\geq 90\%$ Fab or $F(ab')_2$ fragments, and therefore adverse reactions to HBAT are expected to be relatively infrequent (http://www.epi.hss.state.ak.us/bulletins/docs/b2010_05.pdf).

Following formulation of the individual components, the seven antitoxin serotypes are blended into a heptavalent product and filled into single-use vials for intravenous (i.v.) infusion. The nominal potency values for HBAT are: 7,500 international units (IU) anti-A, 5,500 IU anti-B, 5,000 IU anti-C, 1,000 IU anti-D, 8,500 IU anti-E, 5,000 IU anti-F, and 1,000 IU anti-G [56, 91]. These units are more than sufficient to neutralize the highest serum levels of BoNT encountered in natural outbreaks [154]. Because HBAT is despeciated, it is less likely to elicit hypersensitivity reactions [119]. Although it may not be as safe as the human-derived antitoxin approved for infant botulism ([27], Sect. 13.3.4), despeciation should in principle increase the safety of HBAT by removing the Fc region of the IgG which binds complement and triggers inflammatory side effects [154]. Accordingly, skin sensitivity tests that were recommended for the previous formulations of antitoxin are not required for HBAT. Data gathered from 148 BoNT-intoxicated patients treated with HBAT under a CDC expanded access program between 2008 and 2011 suggest

that the new product has similar efficacy as BAT-AB and BAT-E (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/BloodProductsAdvisoryCommittee/UCM338853.pdf). This is based on observations of comparable improvements in the length of hospital stays, time spent in intensive care, and duration of ventilator support with the former and current antitoxins. However, HBAT caused fewer adverse events than had been reported for the former antitoxins, with only one case of serum sickness and no anaphylaxis [120]. This is similar to the safety profile observed with an earlier formulation of HBAT provided by the US Army for a large BoNT/E outbreak in Egypt in 1991 [119].

While despeciation reduced the reactogenicity of the antitoxin, it also shortened its plasma half-life [56]. Perhaps not fully appreciated earlier, the shorter plasma half-life of HBAT can be problematic in cases of intestinal colonization or in wound botulism. A patient with type F intestinal colonization botulism showed initial improvement after HBAT infusion, only to be followed by a relapse 10 days later [91]. The short half-life of type F antibodies in HBAT (14.1 h) was a likely contributing factor since BoNT/F would continue to be elaborated from the gut long after HBAT is cleared from the body. Cases of intestinal colonization and wound botulism will require greater vigilance, with possible repeated infusion of HBAT to prevent recurrence of intoxication [91].

In March 2013, the FDA approved HBAT (BAT™) as an orphan drug for treatment of non-infant botulism in adults and pediatric patients. Unlike previous BoNT antitoxins, which were available in restricted quantities, sufficient doses of HBAT will be stockpiled to meet all expected contingencies. Thus, a total of 200,000 doses will be available in the SNS by 2018. (http://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm345137.htm). HBAT is the first product to receive licensure by the FDA Center for Biologics Evaluation and Research (CBER) under the Animal Efficacy Rule (21 CFR § 601 Subpart H, Approval of Biologic Products when Human Efficacy Studies are Not Ethical or Feasible).

Data in support of licensure of HBAT included safety studies in humans, pharmacokinetic studies in guinea pigs, rhesus macaques, and humans and efficacy studies in BoNT-intoxicated guinea pigs and rhesus macaques. Human efficacy of HBAT was based on the CDC data described earlier under an IND and was also demonstrated in a small-scale study that took advantage of the phenomenon that injection of low doses of BoNT in a restricted volume can produce complete paralysis of the target muscle while avoiding systemic toxicity. This allows for human efficacy studies to be carried out on a Tier 1 agent in a manner that is both humane and ethical (http://ichgcp.net/clinical-trials-registry/research/index/NCT00636519). Extensor digitorum brevis (EDB) muscles of human volunteers were injected locally with 5 MLD_{50} of Botox® (onabotulinumtoxinA) or 500 MLD_{50} of Myobloc® (rimabotulinumtoxinB) by i.m. administration. The experimental group received an i.v. infusion of HBAT 1 day before toxin and exhibited normal EDB muscle function. The "control" group received placebo in place of HBAT and exhibited paralysis of the EDB muscle over the entire 28-day period of observation.

13.3.4 Antitoxin Treatment for Infant Botulism

Infant botulism is currently the most common form of BoNT intoxication in the USA. Unlike classic foodborne botulism, which involves intoxication by preformed toxin, infant botulism occurs when spores of *C. botulinum* (usually A or B) are ingested, and vegetative cells temporarily colonize the large intestine. Under appropriate growth conditions, *C. botulinum* will proliferate and produce toxin that reaches the target tissues via the general circulation [27]. Infant botulism is most frequently observed at 3–4 months of age, and like adult botulism, severe cases of infant botulism require intensive care, artificial ventilation and antitoxin treatment [25].

Equine antitoxin products are not generally used in infant botulism due to their potential for eliciting hypersensitivity reactions, including a lifelong sensitization to equine proteins. Additionally, equine antitoxins have a brief half-life in humans, which is incompatible with the prolonged duration of toxin production in the colonized intestine. To overcome these constraints, a human antitoxin was developed from plasma of laboratory workers who were hyperimmunized with PBT. This product was designated Botulism Immune Globulin Intravenous (Human) (BIG-IV) and contained ≥15 IU of antibodies against BoNT/A and ≥4 IU of antibodies against BoNT/B. BIG-IV was tested in a 5-year randomized double-blind placebo-controlled study in California as well as a 6-year nationwide open-label study. In both studies, treatment with BIG-IV led to significant reductions in mean hospitalization time, including fewer days of intensive care and mechanical ventilation, with no serious adverse effects. The findings were sufficiently compelling that licensure of BIG-IV was granted by the FDA in October 2003 as BabyBIG® for the treatment of infant botulism. Since BIG-IV is derived from human plasma, it also has a long circulation time (mean serum half-life =28 days) and generally remains protective for the duration of the intestinal colonization [27].

13.3.5 Recombinant Monoclonal Antibody-Based Antitoxins

From the above, it is clear that a human product like BIG-IV would be desirable for treatment of adult botulism since it is safe and efficacious, and its long plasma residence time would make it possible to use this antitoxin for pretreatment or prophylaxis. Pretreatment with antitoxin prior to BoNT exposure has been shown to prevent botulism in animals, whereas delayed addition led to a relatively poor prognosis [61, 100, 193]. However, since BIG-IV is derived from hyperimmunized human donors, it would not be feasible to obtain the vast quantities of antitoxin needed for the SNS from such a limited source. Another concern is the problem of screening for infectious diseases, which could become an issue if production of BIG-IV were to be expanded to provide coverage for the general population.

As an alternative to antitoxins derived from either equine or human donors, recombinant monoclonal antibodies could, in principle, provide a sustainable source of antitoxin in unlimited quantity without risk of transmitting infectious diseases.

Marks and coworkers pioneered the expression of "recombinant human BoNT antibodies by phage and yeast display technologies" [20, 174, 195]. These investigators demonstrated that a combination of three monoclonal antibodies produced effective neutralization for a single BoNT serotype [174].

These concepts were recently commercialized by XOMA LLC, who expressed anti-BoNT monoclonal antibodies (mAbs) in Chinese Hamster Ovary cells (CHO) and developed processes to scale up the mAbs for eventual placement in the SNS as next-generation therapeutics, if current licensing efforts prove successful. The product furthest in development is designated as XOMA 3AB and has successfully completed phase 1 clinical trials [37, 161]. XOMA 3AB consists of an equimolar mixture of three IgG mAbs designated NX01, NX02, and NX11, which target different nonoverlapping regions on the HC of BoNT/A; NX01 and NX02 bind to BoNT/A Hc, while NX11 binds to the interface between Hc and translocation domains. These binding sites are highly conserved in BoNT/A subtypes A1, A2, A3, and A4, allowing for one triad of antibodies to effectively neutralize four subtypes of BoNT/A. With respect to other serotypes, a botulism serotype B and E antitoxin combination is in advanced preclinical studies, and antitoxins for serotypes C and D have recently entered initial preclinical testing; antitoxin candidates for serotypes F and G are still under evaluation (http://www.phe.gov/Preparedness/mcm/phemce/Documents/2012-PHEMCE-Implementation-Plan.pdf).

The mAbs are considerably more potent than equine-based antitoxins and have a relatively long half-life of approximately 1 month. In addition, these mABs are expected to produce fewer adverse reactions, due to the absence of heterologous antigens. The latter two attributes may allow XOMA 3 AB to be used prophylactically, if desired, rather than only after signs of exposure are observed. Formulations of recombinantly expressed mAb appear to be highly promising for the production of the next generation of antitoxins for BoNT intoxication; they can provide a more reliable, stable, and sustainable source of antitoxin than is possible with current-generation products.

13.4 Pharmacological Intervention

From the time that inhibition of ACh release was established as the mechanism of BoNT action, attempts have been made to antagonize the neurotoxin by measures that enhance ACh release [165] and, subsequently, with specific inhibitors that target its binding [30], translocation [75] and catalytic activity [78]. The search for inhibitors has intensified during the past two decades, driven largely by the need to provide postexposure protection to both military and civilian populations following the rise in the threat of international terrorism. This pursuit has also been aided by recent advances in our understanding of the mechanism of BoNT action following identification of the specific events between exposure and intoxication [182, 229] and elucidation of the SNARE protein targets of the BoNT LCs [169, 207, 233].

An additional factor in support of a pharmacological approach was alluded to in Sect. 13.1.3 in relation to vaccines. Since the indications for the clinical use of BoNT continue to increase [222, 264], the presence of anti-BoNT antibodies in vaccinated personnel would reduce or abolish the therapeutic benefits of BoNT in these individuals. Although antibody levels decline with time after vaccination to the point of failing to protect against a toxic exposure within 2 years [236], residual BoNT antibodies can still interfere with the therapeutic use of BoNT, perhaps for an entire lifetime. Moreover, this resistance to therapy cannot be overcome by increases in the dose of BoNT [84]. Vaccination with selective serotypes is not practical, since those most likely to be used in a bioterrorist attack (A and B) [26] are also the ones that provide the greatest therapeutic benefit.

The question of whether to vaccinate or not is considered by Simpson [230], who notes that a possible answer may be to develop a vaccine that could produce a rapid and robust immunity to BoNT, but only for a limited duration, corresponding to the period of the heightened threat. Antitoxins, especially the XOMA mAbs, can accomplish this to some extent. However, prophylactic use of antitoxins is precluded since the most likely candidates (XOMA mABs) are still in development, and multiple infusion with equine-derived antitoxins would likely create problems with hypersensitivity [249]. Finally, the experience gained in preparation for a potential BoNT threat during the Persian Gulf War made it clear that delays in generating adequate protection by the BoNT vaccine were not consistent with the requirement for rapid deployment of military personnel [29]. Even the new recombinant Hc vaccine requires multiple vaccinations to achieve protective titers [202], and virally vectored vaccine candidates that are effective after a single injection have yet to transition from basic research to product development [270, 275].

13.4.1 Early Treatment Concepts

Some of the earliest putative BoNT antagonists were cholinesterase inhibitors, based on their ability to prolong the actions of ACh. Carbamate anticholinesterase agents such as neostigmine and physostigmine were investigated in animals [86] and in nerve muscle preparations [112], but they were unable to antagonize the effect of BoNT. More recent studies have tended to confirm earlier findings [4], although there have been reports of patients, especially those with less severe signs of botulism, responding to the short-acting cholinesterase inhibitor edrophonium [62]. Other potential antagonists of BoNT action, such as Ca^{2+} ionophores, La^{3+}, black widow spider venom (BWSV), 2,4-dinitrophenol and agents that raise cyclic adenosine monophosphate (AMP) levels, were also examined for their ability to reverse BoNT toxicity. Evaluation of the above compounds in BoNT-intoxicated nerve-muscle preparations revealed increases in the frequency of spontaneous miniature endplate potentials (MEPPs) but little or no enhancement of evoked endplate potentials (EPPs) or of muscle tensions [70, 228, 251]. Accordingly, they were not considered to be of practical value for treatment of BoNT intoxication.

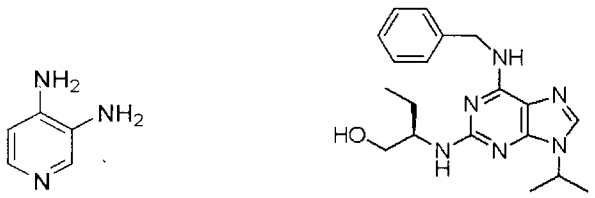

Fig. 13.1 Structure of 3,4-diaminopyrdine (*3,4-DAP; left*) and R-roscovitine (*ROS; right*)

Interestingly, although unable to rescue BoNT/A-intoxicated neuromuscular junctions acutely, BWSV did produce a marked acceleration of recovery of neuromuscular transmission in BoNT/A paralyzed muscles [107]. The acceleration was attributed to rapid destruction of the BoNT/A-poisoned terminals by BWSV, which allowed for reinnervation by a newly formed non-poisoned nerve terminal at the original endplate. In the absence of BWSV, recovery was found to take weeks to months, since the BoNT/A-poisoned terminal does not degenerate but instead prevents nerve sprouts from reinnervating the original endplate. Unfortunately, it has not yet been possible to exploit this phenomenon for accelerating recovery from botulism [160].

13.4.2 K^+ Channel Blockers

K^+ channel blockers were found to be more effective in antagonizing the paralytic action of BoNT than were the former group of compounds. Their higher efficacy comes from their ability to prolong the duration of the nerve terminal action potential [185], leading to a greater influx of Ca^{2+} during nerve stimulation. The increased Ca^{2+} influx enables the K^+ channel blockers to produce striking increases in the amplitude of EPPs and of nerve-evoked twitch tensions [3, 148].

A number of K^+ channel blockers have been evaluated for their ability to antagonize the actions of BoNT, including guanidine, 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP), and tetraethylammonium [63, 64, 70, 148, 164, 165, 227], (see Fig. 13.1). Aminopyridines and tetraethylammonium inhibit different K^+ channel subtypes at the mammalian motor nerve terminal [145, 172, 185], and both compounds are potentially useful for counteracting the inhibitory action of BoNT on transmitter release. Of the K^+ channel blockers thus far examined, the most promising candidate was 3,4-DAP; 4-AP exhibited a higher incidence of central nervous system (CNS) hyperactivity, and tetraethylammonium caused a marked postsynaptic depression of EPPs and nerve-elicited muscle contractions that actually exacerbated BoNT-mediated inhibition after an initial potentiation [3, 4, 228, 251].

When added to nerve-muscle preparations prior to BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions [155, 227]. When applied after BoNT-mediated paralysis, 3,4-DAP was able to augment tensions to or above control values [4, 148, 164, 227]. Unlike most BoNT antagonists, 3,4-DAP could restore tension even in totally paralyzed muscle [5].

In spite of these successes with 3,4-DAP, two fundamental limitations were noted: the efficacy of 3,4-DAP was largely limited to serotype A [227] and the drug had a brief in vivo half-life [5]. Of the two, the latter is less critical since the short duration of action can be offset by the use of an infusion delivery as demonstrated by Adler et al. [9] with subcutaneously implanted osmotic minipumps. In addition, sustained release formulations of the aminopyridine class of K^+ channel blockers have become readily available following introduction of aminopyridines for treatment of diseases such as multiple sclerosis [259].

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. At a functional level, BoNT/A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D, and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPPs [148, 164, 165, 251]. It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release; however, the factors that lead to asynchronous release are still not well understood in spite of dramatic advances in our understanding of the mechanism of transmitter release during the past two decades [72, 197, 203, 242].

13.4.2.1 K^+ Channel Blockers in Human Botulism

The K^+ channel blockers guanidine, 4-AP, and 3,4-DAP have been evaluated in human botulism cases, beginning with an assessment of guanidine in 1967 as an adjunct therapeutic for BoNT/A intoxication. The general findings were that K^+ channel blockers caused a modest increase in the strength of limb, extraocular and postural muscles but these drugs were unable to restore spontaneous ventilation [63, 64, 71].

During the last decade, only a single report was published on the use of K^+ channel blockers in human botulism. In this report, dalfampridine (clinically approved formulation of 4-AP) was examined in a recent case of botulism [120]. A patient admitted originally for cellulitis and treated initially for wound botulism with HBAT and antibiotics was ultimately diagnosed with foodborne botulism. On hospital day 19, the patient was treated with 10 mg dalfampridine administered orally twice a day to reduce muscle weakness and paralysis. Although the patient showed an increase in strength over the following 2 days, the improvement was not attributed to dalfampridine, but rather to spontaneous recovery; the drug was considered to have no effect on this patient.

The basis for the failure of K^+ channel blockers to restore function in respiratory (diaphragm and intercostal) muscles is not known. It is possible that these muscles are inherently less responsive to K^+ channel blockers than limb, extraocular or postural muscles, or respiratory muscles may be more sensitive to BoNT and therefore undergo greater paralysis that is more difficult to reverse. Since systematic dose-ranging studies with K^+ channel blockers were not performed in any of the clinical cases, and high doses were not attempted to avoid the risk of seizures and other

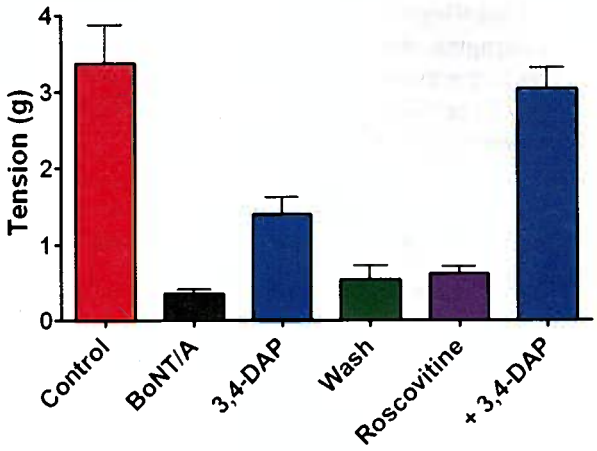

Fig. 13.2 Effect of 3,4-diaminopyrdine (*3,4-DAP*) and R-roscovitine (*ROS*) in reversing botulinum neurotoxin serotype A (*BoNT/A*)-mediated paralysis in isolated mouse hemidiaphragm muscle. Addition of 5 pM BoNT/A depressed the amplitude of indirectly elicited muscle tensions from 3.4 ± 0.5 g to 0.35 ± 0.03 g in 2 h (89.7 % reduction). Addition of 10 μM 3,4-DAP restored tensions to 1.4 ± 0.2 g within 15 min; this effect was reversed by a 30-min washout. Addition of 30 μM ROS produced little detectable increase in tension; however, co-application of 10 μM 3,4-DAP to the 30-μM ROS-containing solution restored tension to near-control values. Symbols represent mean ± SEM; $n = 4$

potential side effects, it is likely that the doses of K^+ channel blockers were not adequate for restoring function in these patients [62, 64, 71, 120]. This certainly appears to be the case for 3,4-DAP, since concentrations required to increase tension in BoNT/A-intoxicated diaphragm muscle were found to be ≥ 10 μM (Fig. 13.2, [13]), and the plasma levels in patients receiving the maximum tolerated dose of 3,4-DAP for conditions such as amyotrophic lateral sclerosis were reported to be almost tenfold lower (1.2 ± 0.5 μM) [16]. Similarly, the dose of dalfampridine in the report of Hill et al. [120] was based on that used for multiple sclerosis and is likely to have been well below the dose required for reversal of BoNT-mediated paralysis [13, 164].

At the present time, the K^+ channel blockers hold promise as potential therapeutic agents, but additional strategies such as development of more selective compounds, targeting of the inhibitors to neuromuscular and neuroeffector synapses or combining these drugs with Ca^{2+} channel activators will be required to exploit their full potential. With regard to more selective inhibitors, Mayorov et al. [155] synthesized new analogs of 3,4-DAP with the goal of finding compounds that displayed both an enhanced affinity for nerve terminal K^+ channels and a reduced propensity to cross the blood–brain barrier. Although none of the analogs was more potent than 3,4-DAP, one was found to have a more favorable peripheral to CNS distribution [155]. Complicating the search for aminopyridines with low CNS toxicity is that their binding site on the K^+ channel is accessible only from the cytoplasmic mem-

brane surface [122, 170]. This makes the goal of finding compounds with reduced CNS penetration challenging, since such compounds would also have an impaired ability to gain access to the cytoplasmic surface of the nerve terminal membrane. For this reason, it may be profitable to focus on K^+ channel blockers that act on the outer surface of the membrane in future studies.

13.4.3 Combination of K^+ Channel Blockers with Ca^{2+} Channel Activators

To address the issue that only high and potentially toxic doses of 3,4-DAP can antagonize the actions of BoNT, we examined the effect of combining 3,4-DAP with the Ca^{2+} channel regulator roscovitine (ROS; Fig. 13.1). ROS, best known as an inhibitor of cyclin-dependent kinases [131], has been evaluated for treatment of human immunodeficiency virus type-1 [110], advanced malignancies [142] and is in phase II trials for non-small cell lung cancer and nasopharyngeal carcinoma [17]. Distinct from its action on cell cycle regulation, ROS has also been found to prolong the open state of nerve terminal N-, P/Q- and R-type Ca^{2+} channels, leading to enhancement of neurotransmitter release [45, 76, 272].

To determine whether ROS could be of benefit in the restoration of tension in BoNT-intoxicated muscles, isolated hemidiaphragms were paralyzed by addition of 5 pM BoNT for 2 h. ROS and 3,4-DAP were evaluated for their ability to reverse paralysis, when added either individually or in combination [11, 13] (Fig. 13.2). Addition of 10 μM 3,4-DAP led to a partial restoration of tension within 15 min of application. Although complete recovery of tension could be elicited with 3,4-DAP alone, higher concentrations of the K^+ channel blocker were required (30–100 μm), which often led to spontaneous muscle fasciculation and multiple twitches following each stimuli [13].

Unlike 3,4-DAP, ROS (30 μM) was not able to reverse BoNT-mediated muscle paralysis on its own. However, when 30 μM ROS and 10 μM 3,4-DAP were co-applied, muscle tensions were restored to near-control levels (Fig. 13.2). As with 3,4-DAP alone, restoration of tension with the combination of 3,4-DAP and ROS was accomplished in ~ 15 min. These results are encouraging since they demonstrate the possibility of achieving a rapid recovery from paralysis by using drugs with synergistic mechanisms of action: increased Ca^{2+} influx via K^+ channel blockade [164] and enhanced Ca^{2+} entry via prolongation of the channel open time [45, 272]. Although the 10-μM concentrations of 3,4-DAP in the combination is still toxic, the concentration of ROS is within the range of plasma levels measured in patients receiving ROS for chemotherapy [156]. By making incremental gains in the margin of safety of the K^+ channel blocker in the combination therapy, it should be possible to achieve efficacy against BoNT in the absence of toxicity. A summary of the role of K^+ channel blockers and Ca^{2+} channel agonists in the treatment of botulism is provided in Table 13.2.

Table 13.2 Candidate pharmacological treatments: Physiological antagonists[a]

Drug candidate	Mechanism	Advantages	Limitations	References
3,4-DAP, 4-AP	K^+ channel blockade	Used clinically	Seizures	[4, 5, 9, 13, 164, 227]
3,4-DAP analogs	Presumed K^+ channel blockade	Reduced CNS penetration	No gain in potency	[155]
Guanidine	K^+ channel blockade	Used clinically	High toxicity	[62, 64]
3,4-DAP + ROS	K^+ channel blockade + increase in Ca^{2+} channel open time	Additive enhancement of agents on Ca^{2+} influx	Toxicity of 3,4-DAP; ROS inhibits cyclin-dependent kinase	[11, 13, 45, 272]

3,4-DAP 3,4-diaminopyridine, *4-AP* 4-aminopyridine, *ROS* R-roscovitine

[a] Advantage of group is rapidity of action and efficacy after intoxication including restoration of muscle tension after total paralysis. Disadvantages of group are that their efficacy is limited to serotype A, and effective concentrations of K^+ channel blockers are toxic in vivo [16]

13.4.4 Inhibitors for Specific Stages of Intoxication

Following the recognition that BoNT enters motor nerve terminal through a series of discrete steps, but prior to establishment of SNARE protein cleavage as the mechanism of BoNT toxicity, attempts were made to develop inhibitors for the binding and internalization of toxin as potential therapeutic candidates. Some of the efforts were intended more to shed light on the mechanisms of action rather than to discover actual treatments, but the search for therapeutics was at least an implicit goal [228]. The current emphasis for therapy is on development of drugs for inhibiting the catalytic activity of the LC, which will be discussed in Sect. 13.4.5, and strategies to accelerate removal or degradation of the LC from intoxicated nerve terminals. The latter is dealt with in Chap. 9 of this volume.

13.4.4.1 Inhibitors of Binding

A reasonable approach to prevent BoNT intoxication is to use receptor antagonists to inhibit the binding of toxin to the nerve terminal. Complications with this approach are that many BoNT serotypes bind to dual polysialoganglioside and protein receptors on the cell surface, and that different BoNT serotypes recognize different protein–ganglioside combinations [134, 167, 274]. This implies that multiple receptor antagonists would need to be developed to protect against all the BoNT serotypes responsible for human intoxications. In addition, although the role of gangliosides in the binding of clostridial neurotoxins was firmly established by the early 1960s [256], the protein receptors for BoNT were not elucidated until 40 years later [79, 80, 134]; by this time, emphasis had shifted to inhibitors of the catalytic activity for potential treatment of BoNT intoxication, as will be discussed in Sect. 13.4.5. The inhibitors of toxin binding that showed the greatest promise were lectins from

Triticum vulgaris and *Limax flavus*, both of which delayed the time-to-block of nerve-elicited muscle contractions with all BoNT serotypes examined [30].

13.4.4.2 Inhibitors of Internalization/Translocation

Following binding of BoNT to receptors on cholinergic nerve terminals, the neurotoxins undergo internalization prior to reaching their ultimate intracellular targets [207, 229, 233]. Internalization is thought to involve endocytosis of the BoNT–receptor complex, acidification of the resulting endocytotic vesicle, dissociation of the LC and HCs, and translocation of the LC into the cytosol [95, 96, 135]. Although it is not known whether LC exists as a discrete entity inside cells or retains some association with other components of the neurotoxin or with cellular components, the isolated LC is known to be the most catalytically active form of BoNT [111].

Translocation affords the next opportunity to ameliorate the toxic actions of BoNT. A number of pharmacological agents have been examined for inhibition of this process with various degrees of success. Simpson [226] demonstrated that pretreatment of phrenic nerve-hemidiaphragm preparations with the lysosomotropic agents ammonium chloride or methylamine hydrochloride delayed the time-to-block of nerve-evoked muscle contractions after exposure to TeNT or BoNT serotypes A, B or C1. Incubation of nerve-muscle preparations with ammonium chloride and methylamine hydrochloride was effective if applied before, concurrently or 10–20 min after toxin exposure. The efficacy of the lysosomotropic agents was reduced rapidly with further delays such that no effect was observed if they were administered ≥30 min after toxin exposure. At optimal concentrations, these compounds produced an ~twofold delay in the time-to-block, but were unable to reduce the degree of paralysis [226].

Other candidates examined for inhibiting BoNT-mediated translocation were the 4-aminoquinoline antimalarial agents, chloroquine and hydroxychloroquine [225]. These drugs were selected on the basis of their ability to accumulate in acidic intracellular compartments and interfere with receptor-mediated endocytosis [268]. The maximal efficacies of the above 4-aminoquinolines were similar to those of ammonium chloride and methylamine hydrochloride, and both groups exhibited a comparable limited therapeutic window. They differed in that effective concentrations of the 4-aminoquinolines also produced a reversible depression of neuromuscular transmission by an unknown mechanism.

Studies on antimalarial agents were extended by Deshpande et al. [75] to identify candidates that did not block neuromuscular transmission, had a longer therapeutic window and could delay the BoNT-mediated time-to-paralysis to a greater extent than the former drugs. These investigators examined a large group of 4- and 8-aminoquinoline compounds as well as analogous acridines for efficacy against BoNT in mouse hemidiaphragm preparations. The most effective compounds were quinacrine and amodiaquine, while 8-aminoquinolines such as primaquine were ineffective. Amodiaquine (20 μM) gave the highest protective index (3.9), defined as the ratio of BoNT-mediated time-to-paralysis in the presence and absence of drug.

Moreover, 20 μM amodiaquine did not impair neuromuscular transmission. The therapeutic window could not be extended, however, and protection was lost if the antimalarial agents were added ≥30 min after exposure to BoNT/A or BoNT/B.

As is clear from the above, strategies targeting BoNT binding or internalization are constrained by a narrow therapeutic window, similar to that which limits the duration of antitoxin efficacy. However, none of the inhibitors of binding and translocation examined to date possess the exquisite potency or selectivity of antitoxins, especially that of the recombinant human mAbs [61, 152]. For these reasons, it is unlikely that pharmacological inhibitors of toxin internalization/translocation will play a prominent role in future drug development efforts.

13.4.5 Inhibitors of Catalytic Activity

The third area for therapeutic intervention is inhibition of the metalloprotease activity of the BoNT LCs. This target is potentially the most promising, since it is not limited by a narrow treatment window as are antitoxins or inhibitors of binding and translocation. In addition, the crystal structures of the LCs for all known serotypes have been solved [14, 24], facilitating the design of antagonists to the active site of the LCs. The presence of a Zn^{2+} binding motif in the LC of clostridial neurotoxins and the finding that Zn^{2+} is required for neurotoxin-mediated proteolysis of SNARE proteins [204] suggest that three classes of potential inhibitors may be effective in antagonizing the toxic actions of BoNT LCs: metal chelators, metalloprotease inhibitors and exosite inhibitors [81, 223].

13.4.5.1 Zn^{2+} Chelators

Simpson et al. [231] demonstrated that the Zn^{2+} chelator *N, N, N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) caused a marked slowing in the time-to-block of nerve-evoked muscle contractions when administered prior to BoNT in phrenic nerve-hemidiaphragm preparations. The maximum efficacy was equivalent to that achieved with *T. vulgaris* lectin, ammonium chloride, methylamine hydrochloride, or the more potent antimalarial drugs.

In common with the above inhibitors, TPEN was effective against all BoNT serotypes examined. In addition, when co-applied with *T. vulgaris* lectin or the lysosomotropic agents, the protection observed with TPEN was approximately additive with that of the former compounds. These results are encouraging since they demonstrate that, in principle, concerted inhibition of the different stages in the production of toxicity is a viable strategy for managing BoNT intoxication. Sheridan and Deshpande [216] examined a number of additional chelators on nerve-evoked twitch tensions and concluded that both a high affinity for Zn^{2+} and membrane permeability were required for antagonism of BoNT.

The efficacy of TPEN in isolated nerve-muscle preparations suggested that it may be able protect mice against BoNT-mediated toxicity in vivo. Thus, TPEN was injected prior to and at 0, 2, 4 and 6 h following a 20-MLD_{50} challenge of BoNT/A or BoNT/B. TPEN prolonged the time to death by 2.1 and 3.3 h for serotypes A and B, respectively. Although TPEN was not able to increase survival, the study clearly demonstrated for the first time that an approach targeting the catalytic activity of BoNT via the active site Zn^{2+} could produce a statistically significant prolongation in the time-to-death [6]. The limitation of TPEN was considered to be its high toxicity; the chelator produced rapid lethality at doses above 30 mg/kg in mice. TPEN was also found to be toxic in primary and clonal cells. TPEN concentrations ≥ 10 μM produced morphological alteration with characteristics of apoptosis and necrosis [8]. Studies with ion replacement indicated that chelation of Zn^{2+} was the proximal cause of cytotoxicity, and examination of a variety of chelators suggested that those with high membrane permeability were especially likely to produce cell death [217].

Based on these findings, metal chelators have little promise for treatment of BoNT intoxication, since the requirements for efficacy against BoNT are the same ones that promote cellular toxicity [6, 7, 217]. These studies did, however, demonstrate that death could be delayed by removal of the active site Zn^{2+} and suggested that inhibition of the catalytic activity in a more targeted fashion may lead to a practical therapeutic.

13.4.5.2 Rationale for Development of Inhibitors Targeting BoNT LC

Since the toxicity of Zn^{2+} chelators discouraged their further consideration, the major emphasis for therapeutic intervention has been focused on metalloprotease inhibitors. This target was selected because of the general consensus that the toxicity of BoNTs stems from the protease activity of the LC [168, 199], and that both peptide and small-molecule inhibitors (SMIs) can, in principle, impede this activity [111, 144].

The basis for targeting BoNT LC with protease inhibitors is to halt SNARE protein cleavage and allow the normal cellular processes to replace cleaved fragments with intact, newly synthesized proteins to promote recovery of function [97]. Moreover, since the time course of BoNT intoxication is relatively long, and the LC persists in a catalytically active state for much of this time [10, 12, 129] ([128], Chap. 9), the LC remains an appropriate target for inhibitors, whereas the binding and translocation domains lose their relevance as therapeutic targets soon after the onset of intoxication [229].

13.4.5.3 Rationale for Focusing on High-Affinity Inhibitors

BoNT/A represents a significant biowarfare and bioterrorism threat [26] owing to two fundamental traits: its unusually high potency, with less than 0.1 μg capable of causing paralysis and death in humans [113], and its long duration of action

following severe intoxication [65, 238, 243]. After exposure to a supralethal dose of BoNT, treatment with 1 μM of a relatively potent LC inhibitor drug of $K_I = 1$ μM would lead to inhibition of only 50% of LC active sites, even if the compound exhibited ideal solubility and membrane permeability characteristics. If a tenfold increase in drug concentration to 10 μM increases inhibition to 90%, the remaining 10% active LC may still be sufficient to maintain paralysis. This is inferred from findings that paralysis in skeletal muscle requires cleavage of only a small fraction of SNAP-25 [125], and it is generally assumed that this can be achieved with relatively few LC molecules per nerve terminal (see however Simpson et al. [232], who suggests that the process of high-affinity binding of BoNT and internalization can produce a higher intraneural LC concentration than generally assumed). Extending our argument further, if raising the drug concentration to 100 μM leads to inhibition of 99% of protease activity, this may perhaps be sufficient to reverse paralysis, if the rates of de novo synthesis of SNAP-25, transport to its presynaptic location and incorporation in the active zone membrane can exceed the residual rate of SNAP-25 cleavage [97].

Based on the above, the currently available BoNT LC protease inhibitors would not be expected to reverse paralysis after intoxication: Problems include low potency, limited membrane permeability, high toxicity and rapid metabolic clearance relative to that of BoNT LC, especially that of the more persistent serotypes A, C1 or B. Several peptide inhibitors have attained submicromolar K_I values for their LC targets [209], but the SMIs discovered to date have not generally reached comparable potencies. The peptide inhibitors, however, cannot readily access LC in the nerve terminal, so that they too are unable to antagonize the action of BoNT in the target tissues [188, 279]. For competitive inhibitors, an additional requirement for high potency stems from the relative abundance of SNAP-25 in the intracellular surface of the presynaptic membrane. It has been shown that BoNT/A LC, once internalized, is localized to the cytosolic plasma membrane within the same subcellular compartment as its target, SNAP-25 [93]. The K_m for SNAP-25 as a substrate for BoNT/A LC is in the range of 0.1–1 μM, depending on experimental conditions. If the local concentration of SNAP-25 within the confined two-dimensional space of the plasma membrane is $10 \times K_m$, the IC_{50} will occur at approximately tenfold higher concentrations than the K_I, since the inhibitor must overcome the high local substrate concentration. This further suggests that competitive inhibitors of the LC will need to be of significantly higher affinity than those currently in existence.

13.4.5.4 Peptide-Based Inhibitors

Peptide-based inhibitors were the first to be examined and remain the most potent in vitro inhibitors of BoNT LC catalytic activity. Shortly after the SNARE protein targets of the BoNTs and specific toxin cleavage sites were elucidated [36, 203, 204, 205, 271], the requirements for substrate recognition by the LCs were systematically investigated. Schmidt and Bostian [208] synthesized a series of short peptides based on the SNAP-25 sequence flanking the BoNT/A cleavage site (residues

197–198) and determined the minimal amino acid sequence required for enzymatic activity. These authors found that short peptides corresponding to residues 187–203 of the 206 amino acid SNAP-25 were sufficient for cleavage by BoNT/A LC; truncations past the P7-Arg or the P5′-Met led to significant reductions in activity. Additional work revealed that certain amino acid modifications, especially at the P2, P1′, and P2′ positions of the peptide, disrupted substrate cleavage [208]. These efforts led to the development of a series of peptide inhibitors based on the P1 through P6′ residues of the native SNAP-25 sequence in which four different amino acids were changed to Cys [212]. The most potent of these inhibitory peptides, Ac-CRATKML-amide, was found to have a K_I of 2 μM and has served as the template for subsequent peptide-based BoNT/A LC inhibitors.

The effect of Cys residue replacement at the P1 position on activity suggests that the high affinity of Ac-CRATKML-amide was a result of the thiol group forming a complex with the active site Zn^{2+}, a suggestion later verified by crystallographic data [224]. In subsequent studies, Schmidt and colleagues modified the Cys group to unnatural thiol-containing side chains [209]. The first observation made was that a single carbon separation between the thiol and the carbonyl group resulted in approximately tenfold higher affinity relative to that found with a two-carbon separation. The resulting 2-mercapto-3-phenylpropionyl (mpp)-RATKML-amide peptide had a K_I of 330 nM.

A second structure–activity relationship (SAR) study using this same mpp group coupled to amino acid chains of different lengths showed that mercapto-peptides, when truncated from the C-terminus of mpp-RATKMLGSG, underwent a tenfold decrease in binding affinity if the C-terminal five residues (MLGSG) were removed. An additional tenfold reduction was observed when a Lys residue was removed, thus confirming the importance of the P5′ and P6′ residues in both inhibitor and substrate binding [209]. The success of the thiol peptides as inhibitors of BoNT/A LC led to a similar approach for the development of inhibitors for BoNT/B [176] and for BoNT/F [211].

Following publication of the seven residue inhibitory peptides by Schmidt and colleagues, the scaffold was modified extensively by various investigators. It was concluded from the crystallographic and biochemical studies of peptides–LC interactions that the active site of BoNT/A is large and flexible, and capable of binding peptides with significant sequence modifications, albeit with affinities ranging from mid-μM to mid-nM. The most potent peptide inhibitor of BoNT/A thus far published was synthesized by Axel Brunger's group [278], and was also based on the Ac-CRATKML-amide scaffold. In their SAR study, 13 peptides were assayed for inhibitory activity with systematic modifications at the P1, P2′, and P4′ positions. The greatest increase in affinity occurred when the P2′ Ala residue was changed to the larger aromatic amino acid Trp or to the unnatural benzothien-3-yl-alanine moiety. Less significant increases in affinity were observed when the P1 residue was changed to the large dinitrophenyl-2-aminobutanoic acid, and the side chain or P4′ Lys residue was shortened to 2,4-aminobutanoic acid. The effect of the modifications was essentially additive, resulting in a K_I value of 41 nM [278]. Co-crystals of the peptide with BoNT/A LC demonstrated a partial helical structure of the peptide

within the active site of BoNT/A LC. Comparable binding orientations were observed in a subsequent study with three additional peptides [279]. Swaminathan and colleagues made similar observations in their co-crystals of the weak 6-mer inhibitors QRATKM and RRATKM (IC_{50} = 133 μM and 95 μM, respectively), i.e., that the inhibitor peptides adopt a more ordered, slightly helical structure within the active site of BoNT/A LC [136].

The Arg residue of the P1′ position is critically important for substrate cleavage as well as peptide inhibitor binding affinity for BoNT/A LC, a property unique to this serotype. Kumaran et al. [136] reported that poly-Arg peptides were able to inhibit the BoNT/A LC. This led to a set of four peptides with the sequence RRGx, where x was C, I, L, or M. The peptides all inhibited the BoNT/A LC with K_I values slightly < 1 μM [136]. Crystal structures of the inhibitors with BoNT/A LC revealed that the N-terminal Arg occupied the P1 position and the second Arg occupied the P1′ position, i.e., the expected location of the P1′ Arg in wild-type SNAP-25.

When the peptide–BoNT LC crystallographic data are considered as a whole, an additional noteworthy observation can be made: The orientation of the Ac-CRATK-ML-amide peptide within the active site is not the same as in the other peptide crystal structures [224]. In the former, a complex is formed between the active site Zn^{2+} and the thiol group of the P1 Cys residue, or more accurately, the sulfenic acid group [224]. For all other peptide inhibitor structures, the complex is produced by the backbone nitrogen and carbonyl oxygen forming the amide bond between the P1 and P1′ residues. Thus, although the Ac-CRATKML-amide and the other peptides adopt a partial helical secondary structure within the LC binding site, the Ac-CRATKML-amide is oriented one amino acid out of register with respect to the other peptide inhibitors (cf. [279]).

Other methods for discovering higher affinity peptides have included phage and mRNA display. Phage display represents the earliest method for obtaining directed libraries of BoNT inhibitors [88]. More recently, the same technique has been used for the development of camelid antibodies targeting BoNT holotoxin [106]. The related mRNA display method achieved similar results, although the selection process was performed entirely in vitro and was thus not dependent on expression on the surface of a biological entity. This technique identified peptide inhibitors with a five amino acid N-terminal extension on the parent CRATKML peptide, possessing lower IC_{50} and K_I values for BoNT/A LC than the parent peptide [273].

13.4.5.5 SMIs: Hydroxamates

Although potent peptide inhibitors were successfully developed for several BoNT serotypes, the general lack of stability and poor membrane solubility of peptides [258] has directed the majority of BoNT inhibitor efforts toward SMIs. The hydroxamic acid, or hydroxamate moiety, is a simple chemical structure that can form stable complexes with Zn^{2+} and is frequently found in metalloprotease inhibitors. The small size of hydroxamates permits them to be added to more complex structures. In addition, hydroxamates are uncharged at physiological pH, allowing them

potential access to the intracellular environment. The first study using a hydroxamate for mitigating BoNT intoxication was performed by Deshpande et al. [74] and revealed no antagonism of hemidiaphragm paralysis following BoNT/A or BoNT/B exposure. A decade later, Janda and colleagues performed a series of systematic studies on hydroxamates. In a first step, Arg-hydroxamate was synthesized and found to be a weak inhibitor of BoNT/A LC (K_I = 60 μM) using a 66-mer SNAP-25 substrate and a truncated recombinant BoNT/A LC [38]; further derivations of Arg-hydroxamate did not improve potency.

A second study was carried out using a recently developed process to convert a series of off-the-shelf carboxylic acids into hydroxamates [121]. This resulted in several compounds that were able to inhibit BoNT/A LC-mediated cleavage of the substrate SNAPtide® measured using Förster resonance energy transfer (FRET) spectroscopy [39]. One of the lead compounds, *para*-chloro-cinnamic hydroxamate, was further derivatized to yield 12 additional compounds. The most active of these was *ortho-para*-dichloro-cinnamic hydroxamate (or 2,4-dichloro-cinnamic hydroxamate, DCH), with an IC_{50} of 410 nM.

An additional hydroxamate, 1-adamantane-*N*-hydroxyacetamide, was also identified using similar techniques [54] In an attempt to displace an active site water, as well as to determine the importance of chirality, a hydroxyethyl moiety was attached to the central carbon chain of DCH in a stereoselective manner, thus introducing a chiral center in this hydroxamate [245]. The (R)-enantiomer of the molecule gave a K_I of 160 nM, fourfold lower than the (S) compound and twofold lower than the unsubstituted DCH. Additional hydroxamate-based inhibitors with markedly different structures yielded K_I values of 5–6 μM using a similar SNAPtide assay and truncated recombinant BoNT/A LC from List Biologicals [253]. A caveat for enzymatic studies using truncated BoNT/A LCs is that the absence of residues 425 through 437 at the C-terminus can make the truncated LCs more susceptible to SMIs than the full-length LC and also more sensitive to variations in assay conditions. For example, although DCH was initially reported to have an IC_{50} of 410 nM, [39] was subsequently found to have a much higher IC_{50} of 59 μM [49] or 81 μM [187] under different assay conditions.

Computer-aided molecular design has generated several novel parent structures for BoNT/A LC inhibitors. Using a library of 2.5 million compounds and the crystal structure of the BoNT/A holotoxin complex [138], an in silico screen was performed using the cationic dummy-atom approach [179] to better estimate the BoNT/A active site Zn^{2+} binding affinity of the compounds [184]. The initial in silico screen yielded eight "hits," all of which were screened in an HPLC BoNT/A LC inhibition assay using a 17-mer SNAP-25 peptide as the substrate. One of the eight compounds, [5-(4-chlorobenzoyl)-2-phenylthiophene-3-yl]acetic acid, produced 15% inhibition at 100 μM and was selected for further derivatization. Seven additional derivatives, four of which possessed hydroxamate moieties, were synthesized and tested but showed little increase in activity. Replacing 4-chlorobenbenzoyl with a phenyl-indole-carbonyl group resulted in only 4% inhibition at 100 μM. However, subsequent addition of an amino group to the indole nitrogen of the latter compound connected by four, five or six methylenes resulted in 96% inhibition at

100 μM. The *N*-amino substituted phenyl-indole-carbonyl-phenylthiophene-3-yl-hydroxamate compounds were tested and found to have similar activity, with the best compound giving a K_I of 12 μM [184]. Addition of a hydroxyl to the phenyl of the phenylthiophene-3-yl group resulted in a threefold decrease in the K_I, as well as complex inhibition patterns at high concentrations [250].

Using this latest structure as a scaffold for derivatization, additional changes were made: In two compounds, the hydroxyl on the phenyl-thiophen-3-yl was changed to an amine, and in a third compound, the hydroxyl was moved from the *meta* to the *para* position [181]. At 20 μM, the three compounds designated as H3H, F3A, and F4H inhibited BoNT/A LC activity by 78, 47 and 82 %, respectively, in an HPLC assay. Interestingly, these three compounds showed some protective effect against BoNT/A intoxication in vivo as will be discussed in Sect. 13.4.5.6.

Based on their success in the development of the phenylthiophenyl hydroxamates, additional inhibitors were synthesized by Pang and colleagues using a system termed synthesis-based computer-aided molecular design (SBCAMD). In this process, computer-aided design of novel derivatives is integrated with existing organic synthesis capabilities, resulting in molecular design of compounds that can be readily synthesized and tested for inhibition of BoNT/A LC [180]. After synthesis and assay of nine novel compounds of diverse structures, the most potent was a hydroxamate comprised of bis-7-aminoheptyl, diphenylpropyl, and phenylpyrole substituents with a K_I of 760 nM.

13.4.5.6 In Vivo Hydroxamate Studies

BoNT/A is known to have an extremely long duration of action [129], a property likely arising from the ability of the BoNT/A LC to evade intracellular degradation processes [255] ([137], Chap. 9). It is also known that upon systemic exposure to BoNT/A, the toxin can remain within the vasculature for several days [115, 139, 193, 249]. The protease inhibitors will typically be cleared from the body on a timescale of hours to days, while the target enzyme will in principle remain in nerve terminals for months. Thus, for SMIs directed against BoNT/A LC protease activity, a single dose administered at the same time as toxin would not likely result in complete reversal of effects, due to the considerable differences in pharmacokinetic timescale between the two entities. Once the inhibitor is eliminated, the LC will continue to cleave SNAP-25, and any observable improvement of paralysis would rapidly disappear.

The typical experimental paradigm used to determine in vivo efficacy of BoNT/A inhibitors is to administer a high dose of compound into the tail vein of mice. Immediately following, or up to 30 min after the inhibitor is given, animals are challenged with a 5–10-MLD_{50} dose of BoNT/A. Assuming that the pharmacokinetics of a hypothetical SMI follow simple first-order clearance with a hypothetical half-time of 6 h, < 10 % of the compound would be expected to remain after 24 h and < 1 % after 48 h. If a 10 μM (i.e., $10 \times K_I$) intracellular concentration were achievable for the hypothetical compound, this concentration would only exist transiently, becoming

reduced to its K_I value within 24 h. This would be followed by recurrence of SNAP-25 cleavage and paralysis.

With these concepts in mind, several studies evaluated compounds for protection against supra-MLD_{50} doses of BoNT/A with paradoxical results. An early example of this was a study performed on the DCH compound. Mice were injected i.v. with 0.1 ml of a 1-mM DCH solution into the lateral tail vein followed immediately with an i.p. injection of a 0.5-ml solution of a 5–10-MLD_{50} dose of BoNT/A. Of 31 mice injected, 5 survived the BoNT/A challenge indefinitely with no observable ill effects [89]. The other 26 mice died with a time course similar to that of vehicle-treated BoNT/A-injected mice. This apparent protective effect of DCH would not have been predicted from cell-based assays because DCH had been reported to be cytotoxic in cellular models. An additional nonhydroxamate compound described in the study prolonged the time-to-death by 36%, with no subpopulation of mice seemingly unaffected by the toxin, a result much more in line with expectations.

A comparable finding was reported by Pang et al. [182] for the indole phenyl-thiophen-3-yl hydroxamates described earlier. Increased survival was observed in mice pretreated with 2 mg/kg of inhibitor followed 30 min later by i.p. challenge with 5 MLD_{50} of BoNT/A; five of ten animals were alive 24 h after BoNT/A, whereas none of the five vehicle-treated animals (DMSO) were able to survive 5 MLD_{50} of BoNT/A. As with DCH, one of ten mice in each compound-treated group survived BoNT/A challenge with no signs of intoxication for up to 5 days posttreatment.

In the study of Pang et al. [181], the pharmacokinetics of the compounds were also examined. Following a 2-mg/kg i.p. injection of each compound, plasma half-lives were observed to range from 4.4 to 6.5 h for the three compounds. Maximum plasma concentrations (C_{max}) ranged from 256 to 738 ng/ml (0.46–1.32 μM). Although the C_{max} does not necessarily reflect the concentration inside the nerve terminal, it is doubtful that the latter would accumulate the inhibitor at concentrations significantly higher than the C_{max} in the absence of an active transport or retention mechanism. Since these compounds were reported to inhibit 47–82% of BoNT/A LC activity at 20 μM (a concentration >20-fold higher than the C_{max}), it is unlikely that the compounds exerted their effects in vivo by inhibition of BoNT/A LC activity in the nerve terminal; the more plausible hypothesis is that these hydoxamates are acting on toxin in the extracellular compartment, i.e., by inactivating or increasing clearance of the toxin before BoNT/A LC internalization.

These results suggest a complex mechanism of action for BoNT/A LC inhibitors in vivo. Despite the demonstrated inhibition of BoNT/A LC protease activity in vitro, the pharmacokinetic and pharmacodynamic data are difficult to reconcile with protease inhibition as the dominant mechanism. The duration of action of the BoNT/A LC is orders of magnitude longer than the plasma half-life for any of compounds examined, so that long-term survivors following a single pretreatment with a protease inhibitor would not be expected in animals challenged with multiple MLD_{50} doses of BoNT/A. Furthermore, the concentrations of drug required for inhibition of BoNT/A LC in vitro do not appear to be reached in vivo. Additional studies in a true postexposure model in which the inhibitor can be applied after

intoxication over an extended time frame via multiple injections or continuous minipump infusion may help to unravel the paradox.

13.4.5.7 Mercaptoacetamides

In an effort to develop additional scaffolds for SMIs of BoNT/A, a mercaptoacetamide structure–activity study was performed [163]. These compounds were synthesized as an extension of the work on Ac-CRATKML-amide, which utilizes the free thiol group of the N-terminal Cys to form a complex with the active site Zn^{2+}. As was observed by Schmidt and Stafford [209], a single carbon between the thiol and carbonyl groups was optimal for inhibition. Derivatives of the phenyl-pyrazole ring resulted in multiple compounds with IC_{50} values < 100 μM, with the three best compounds ranging from 3 to 7 μM. These compounds were all similarly substituted at the 4-phenyl position and were active both in rat primary cerebellar neurons and, to a limited degree, in a mouse phrenic nerve-hemidiaphragm preparation [163].

13.4.5.8 Bis-Imidazoles

Based on the idea that imidazole moieties readily form complexes with Zn^{2+}, Merino et al. [158] performed a series of experiments exploring bis-imidazoles linked by carbon chains of varying lengths. A preference of linker chains comprising 13 methylenes was observed, with molecular modeling showing the dual imidazole groups spanning the active site Zn^{2+} and Glu54. Methylene chain lengths of 12–16 gave essentially the same percent inhibition (maximum 61 % at 100 μM), with chain lengths of 9–11 giving weaker inhibition. Furthermore, the study found that imidazoles linked by bis-amide-methlylene chains had essentially no activity, suggesting that inhibition may have resulted from hydrophobic interaction within the binding site rather than from efficient spanning of the Zn^{2+} and Glu54 by the imidazole.

13.4.5.9 Multi-Zone Pharmacophore Models

The initial attempts at structure–activity studies in developing inhibitors to BoNT LCs were hampered by a lack of high-resolution structural data for the LCs complexed with either native substrate or inhibitor. Initial molecular models were built using crystallographic data for the BoNT/A holotoxin [138]. In an effort to incorporate existing and emerging biochemical data into a structural model for BoNT/A LC inhibitors, Burnett and colleagues have developed a continually evolving pharmacophore comprised of multiple zones in which a chemical moiety is positioned in a three-dimensional (3D) scaffold [50, 51]. This model has led to the development of several distinct compounds with related structural motifs.

The initial data for the development of the pharmacophore model began with a screening of the National Cancer Institute (NCI) Diversity Set consisting of 1,990

compounds composed of a wide range of structures. Compounds were screened for inhibitory activity using the full-length recombinant BoNT/A LC [15] and a short peptide-based fluorescent substrate [210, 213], with the initial hits verified by an HPLC-based assay. A total of 21 compounds were studied [47]. Two compounds were 8-quinolinols, a known Zn^{2+} chelating compound, and the inhibitory capacity of these compounds was found to be reversed by 20 μM $ZnCl_2$, thus disqualifying them from further study.

Subsequently, Burnett and colleagues examined existing libraries of *N,N*-bis(7-chloroquinolin-4-yl)alkanediamines and heteroalkane diamines developed initially as antimalarial agents [257]. In addition, several clinically used antimalarial compounds were tested. Although most were weak inhibitors of BoNT/A LC at 20–50 μM, several of the "hits" from both screens were used to develop their first pharmacophore model. In this model, flexible linkers of seven or more intervening methylenes or amines allowed the two bisquinoline moieties to fold into a structure consistent with the other compounds, forming a common pharmacophore [47]. The compounds Q2–15 and michellamine B were the most potent, and an additional series of molecular dynamics and docking simulations was performed using the existing crystal structures of the BoNT/A holotoxin [138, 214]. The dynamics simulations showed little change in the secondary structure of the BoNT/A LC, but did reveal large changes in the surface loops, referred to as loop 1 (residues 48–78), loop 2 (residues 167–180), and loop 3 (residues 232–258). The contact points between the individual inhibitor compounds and the structural model of the LC were used to modify the pharmacophore model [48].

The progression of a molecular model is dependent on continual refinement with biochemical data. To this end, the previously described peptide mpp-RAT-KML was docked into the active site of the BoNT/A LC crystal structure [214]. However, the authors found that the docking orientation did not satisfy SAR experiments previously published [209]. In particular, the dramatic loss in activity when the Arg was changed to a Lys could not be explained unless the loop 1 residues 48–78 were reoriented. When the pharmacophore was expanded to incorporate chemical moieties not included in the initial model, several new inhibitors were found, with four possessing K_I values of 3–10 μM. Three compounds were observed by autofluorescence to be rapidly taken up by primary cultures of chick neurons, although two of the compounds were cytotoxic at low micromolar concentrations. One compound, NSC240898 (2-(4-(4-(aminoiminomethyl) phenoxy)phenyl)-1H-indole-6-carboximidamide), had a K_I value of 4.6 μM as determined by isothermal titration calorimetry and was less cytotoxic than the other compounds. In addition, NSC240898 showed dose-dependent protection of endogenous SNAP-25 cleavage. Chick primary neurons were incubated for 30–45 min with 5–40 μM NSC240898, then for an additional 3.5 h with 10 nM BoNT/A in the continued presence of inhibitor. Densitometric analyses showed a small dose-dependent decrease in cleaved SNAP-25. However, since the toxin and inhibitor were co-applied to the cultured neurons, the mechanism of action of the inhibitor cannot be differentiated among blockade of entry, disruption of intracellular translocation or inhibition of the protease activity. Furthermore, the authors used a

much higher dose of BoNT/A (10 nM) than is typical for intoxication of primary neurons. This could lead to a very high intracellular copy number of BoNT/A LC requiring an inhibitor concentration significantly higher than its K_I value to adequately block the LC activity [49].

The pharmacophore model was used to reexamine the 4-amino-7-chloroquinolines identified previously from bis-quinoline libraries [47], with the refined pharmacophore model used to develop additional derivatives. The authors replaced one of the chloroquinoline moieties with a congeneric series of cholate acetates and tris-chloroquinoline, identifying three new inhibitors with IC_{50} values ranging from 3.2 to 17 μM. As with previous molecular models, the chloroquinolines fit into the BoNT/A LC subsite S1′, while the cholate portion occupied the LC substrate cleft and positioned functional groups into subsite 2′ [50]. An additional series of 4-amino-7-chloroquinolines coupled to steroidal and adamantane constituents provided compounds with IC_{50} values of 12–50 μM for inhibition of BoNT/A LC. Linking the cholate or the adamantine groups to the chloroquinoline moiety via two- or three-carbon linkers resulted in compounds with inhibitory activity, whereas longer linkers or linkers possessing an amide bond had no activity, as was also found for compounds with a second adamantane moiety [240].

Continuing to expand on the three-zone pharmacophore model, a 4-amino-7-chloroquinoline group was added to each end of the existing aminoiminomethyl-phenoxy-phenyl-indole carboximidamide of the compound NSC240898, resulting in an analog with a K_I of 600 nM [51]. The improved K_I resulting from addition of the chloroquinoline group to the existing amino-phenoxy-phenyl indole structures led to the hypothesis of a four-zone pharmacophore in which chloroquinoline groups were added to each end of an additional structure identified via the 3D database screen. These compounds possessed IC_{50} values of 600–900 nM [175].

In a departure from the pharmacophore model, two compounds identified by Burnett et al. [50] were used as query structures to perform a 3D database search of the ~ 270,000-compound NCI Open Repository. Twenty "hits" were mapped to the search query, of which ten were available for testing; three inhibited BoNT/A LC in the standard HPLC assay. Two of the compounds were congeners of the query compounds, as would be expected in a 3D database search. A third compound possessed a fused four-ring diazachrysene scaffold reminiscent of the cholate structure used in their previous work, although the diazachrysene is aromatic and more structurally rigid than the cholates.

The three compounds produced 40–50% inhibition of BoNT/A LC activity at 10 μM and thus would have presumed IC_{50} values of ~ 10 μM. The authors also identified several closely related compounds that were not active, demonstrating the specificity of active site fit required for inhibition and arguing against nonspecific effects of the compounds. A second query resulted in two additional compounds with similar activities and unique structures [117]. The 1,7-bis(alkylamino)-diazachrysene structure identified from the NCI screen was further derivatized via modification of the bis-alkylamino substituents. Thirteen derivatives inhibited BoNT/A LC 39–73% at 20 μM. Interestingly, the compounds also showed activity as both antimalarials and antivirals [177].

13.4.5.10 Quinolinols

Using slightly different docking parameters, Roxas-Duncan et al. [200] also used the NCI compound database to perform an in silico screen for compounds capable of docking into the BoNT/A LC active site based on the unliganded BoNT/A LC crystal structure [214]. The database screen yielded ~ 500 candidate compounds, of which 100 compounds that fit best into the active site were chosen for biochemical screening. Of these, seven inhibited LC activity at 20–200 μM. An 8-quinolinol compound, NSC1010, was chosen for further study. Although NSC1010 was the most potent inhibitor of the group, it was found to be toxic to cultured cells and was instead used to perform a similarity search of compounds in the NCI, Sigma, and ChemBridge compound databases. An additional 55 compounds were identified and tested in the same biochemical assay, and the five most active compounds, all 8-quinolinols, were chosen for additional study. IC_{50} values for the group ranged from 1.5 to 5.0 μM when tested with either the full-length or truncated BoNT/A LCs. All five of the compounds had significantly less cellular toxicity despite being more potent than NSC1010 in inhibiting BoNT/A LC [200].

The authors tested their hits in cell-based and muscle-function assays. In the former, Neuro2A cells were completely protected from BoNT/A-induced cleavage of endogenous SNAP-25. However, the assay was performed by preincubating BoNT/A holotoxin with inhibitor for 30 min at 37 °C. Since incubations were carried out before BoNT/A was exposed to cells, it is difficult to distinguish whether the effects are occurring inside or outside of the cell, and thus it is not clear if the compounds have promise as post-intoxication treatments. Similarly, mouse hemidiaphragm muscle twitch studies showed an apparent antagonism of BoNT/A activity, but only when the compound and toxin were incubated in advance of applying to the muscle bath. The authors reported that pretreating cells or hemidiaphragm preparations with inhibitor before addition of toxin did not protect either from BoNT/A intoxication [200].

In a recent study, quinolinols were investigated using SNAP-25 from rat brain synaptosomes as substrate for BoNT/A LC [234]. SNAP-25 was considered to be more relevant than small synthetic peptides since the former, like the natural substrate, was full length and membrane bound. Three compounds from the NCI and ChemBridge libraries were found to be sufficiently potent to transition to in vivo studies. One compound (NSC 84087) provided substantial protection of SNAP-25 at 100 nM and extended survival from ~ 9 h to 48 h in mice challenged with BoNT/A (5 MLD_{50}). NSC 84087 was equally effective when co-administered with BoNT or applied 30 min after toxin. NSC 84087 was somewhat less effective when animals were pretreated with inhibitor for 30 min prior to BoNT challenge. These results are of considerable interest, since NSC 84087 is the first inhibitor that has been found to be effective when administered after intoxication, and greater than fivefold increase in survival time is the most dramatic in vivo protection reported to date.

13.4.5.11 Exosite Inhibitors

Recognition between BoNT/A and SNAP-25 involves interaction of substrate with two spatially distinct exosites (α and β) as well as with the active site [42]. Although small peptides such as SNAPtide (13-mer) or the 17-mer peptide used in inhibitor screens can be cleaved by BoNT/A LC, full activity requires the presence of ≥66 residues (141–206). This substrate requirement is unique for BoNT and generally not seen with other Zn^{2+}-containing endoproteases such as thermolysis [190]. The large interaction area between SNAP-25 and BoNT/A LC as seen in the co-crystallization studies, coupled with the biochemical data indicating that full catalytic efficiency only occur when nearly all of the interacting regions of SNAP-25 are present [201], suggests that inhibitors that bind tightly to exosites on the BoNT LC could potentially inhibit substrate binding from outside of the catalytic site [81]. These exosite inhibitors could be co-administered with an active site inhibitor to produce an additive effect that could potentially be more effective than either inhibitor alone. Alternatively, the combination of exosite and active site inhibitors may enable use of lower concentrations of each, allowing for a reduction in off-target effects.

Recently, several potential lead compounds for exosite inhibition have been identified. D-chicoric acid, a component of Echinacea, was found to be a noncompetitive inhibitor of BoNT/A LC [223]. D-chicoric acid was also determined to be a noncompetitive inhibitor of BoNT/B LC, but only if a larger FRET substrate was used (58-mer); no inhibition was observed with shorter substrates, suggesting that the longer substrate was required for the chicoric acid binding site. A similar observation was made when comparing SNAPtide with a 66-mer SNAP-25 peptide as substrates for BoNT/A LC; only the latter was inhibited by chicoric acid.

Lomofungin, a broad-spectrum antibiotic, was identified from the Johns Hopkins Clinical Compound Library of ~ 1,500 compounds, using a high-throughput fluorescence screen. As with chicoric acid, a secondary biochemical assay using SNAP-25 (141–206) demonstrated lomofungin to have a K_I of 6.7 μM, with classical noncompetitive inhibitor kinetics [90]. Additional experiments suggested that lomofungin was mutually nonexclusive in binding with both DCH (competitive inhibitor) and chicoric acid (noncompetitive inhibitor), thus revealing three distinct sites on BoNT/A LC targetable with inhibitors. These findings expand the potential armamentarium for attack on the BoNT/A LC.

13.4.5.12 Irreversible Inhibitors

Because of their extraordinarily high potency and long duration of action, irreversible inhibitors may be more effective in antagonizing BoNT intoxication than competitive inhibitors. Although inhibitors functioning via covalent modification have the potential for significant off-target effects, an antagonist of sufficient potency and specificity could be administered in lower doses for a shorter period of time to an intoxicated individual to mitigate any off-target complications.

With this in mind, Janda and colleagues have taken steps toward development of an irreversible inhibitor for BoNT/A LC by replacing the hydroxamate moiety of DCH with either a cyclopentenedione or a maleimide group [54]. To assay for inactivation of the LC, different concentrations of compounds were incubated with BoNT/A LC for fixed time intervals. The enzyme–inhibitor mixture was diluted 100-fold with 10 μM SNAP-25, and aliquots were assayed at regular time intervals. Catalytic rates were determined from the HPLC peaks of the cleaved 9-mer fragment at each time point.

Their results demonstrated enzyme inactivation in cell-free assays and in a primary neuron assay of SNAP-25 cleavage. In each case, high concentrations of inhibitor were required, and the primary neuron assay was performed as a co-incubation of the toxin with the inhibitor. Thus, it is still unknown if the compounds are capable of reversing the effects of BoNT/A after intoxication. The study, however, serves as a proof of concept for the potential role of irreversible inhibitors in the antagonism of BoNT intoxication.

13.4.5.13 Natural Product: Toosendanin

Toosendanin is a triterpenoid compound obtained from the bark of *Melia toosendan* and has been used in traditional Chinese medicine as an antiparasitic agent and agricultural pesticide. Toosendanin was reported to inhibit BoNT intoxication in animal models, including nonhuman primates, and to alter the action of a number of ion channels, including the BoNT/A translocation channel [143, 218]. Based on reports of its efficacy against BoNT in the Chinese literature, considerable interest has arisen in toosendanin as a potential BoNT antagonist [2, 78]. In our laboratory, we have found that toosendanin was able to antagonize BoNT/A intoxication in isolated mouse phrenic nerve-hemidiaphragm preparations when administered at the same time or 30 min before BoNT/A, but not when given 30 min after BoNT [2]. We have also found that toosendanin is equally effective against BoNT serotypes /A, /B or /E. The absence of serotype selectivity, coupled with its reported action on the BoNT translocation channel [96], suggests that toosendanin may be acting on a common step such as slowing translocation of the LC into the nerve terminal cytosol. Current efforts are focused on examining analogs of toosendanin to shed more light on its mechanism of action.

13.4.5.14 Insulin-Like Growth Factor

Intoxication by BoNT/A leads to flaccid paralysis that can last for many months, leading to extensive remodeling of neuromuscular junction. Recovery is delayed by the continued persistence of BoNT LC proteolytic activity [129], the inability of newly formed nerve sprouts to innervate the original endplate, and the extensive loss of muscle protein. We have demonstrated that injection of the insulin-like growth factor 1 (IGF-1) in BoNT/A-paralyzed rat *extensor digitorum longus* (EDL) muscles led to marked improvements in twitch and tetanic tensions (Fig. 13.3) and

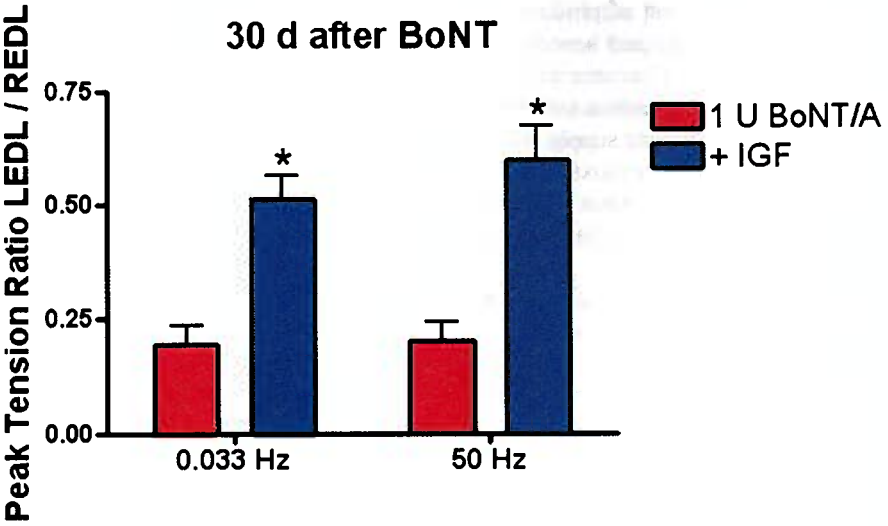

Fig. 13.3 *Extensor digitorum longus* (*EDL*) muscles were injected locally with 5 mouse intraperitoneal median lethal dose (MLD_{50}) of botulinum neurotoxin serotype A (*BoNT/A*) (15 µl) at day 0, followed by local injections of insulin-like growth factor 1 (*IGF-1*) (*blue*) or saline (*red*) twice per week for 30 days in the same muscle. At the end of this time period, muscles were tested in situ for twitch (0.033 Hz) and tetanic (50 Hz) tensions following stimulation of the peroneal nerve. Injection volumes of IGF-1 or saline were 50 µl. In the absence of IGF-1, muscle tensions (twitch and tetanic) recovered to only ~20% of control at 30 days, but increased to more than 50% of control in IGF-1-treated muscles. These differences were highly significant (*$p<0.001$). The bars represent mean ± SEM, $n=6$. LEDL/REDL is the ratio of tensions in the left EDL (BoNT/A-injected) to right EDL (control) muscles

muscle mass compared to BoNT-intoxicated muscles treated only with vehicle. The mechanism of IGF-1 in protecting muscles from BoNT-mediated paralysis is unknown. Possibilities include (1) enhanced sprouting of preterminal nerve fibers leading to hyperinnervation of muscles beyond the original intoxicated endplate, (2) increased rate of recovery of function at the original endplate or (3) direct actions of IGF-1 on muscle fibers to enhance muscle mass. The data in Fig. 13.3 were obtained using a locally injected model of BoNT intoxication [5]. Based on these results, IGF-1 may also be expected to accelerate recovery following systemic BoNT intoxication, especially when combined with an effective inhibitor of LC-mediated proteolysis.

13.5 Conclusions and Future Research

Substantial progress has been made in transitioning from the traditional toxoid vaccine for BoNT intoxication to the development of safe and effective recombinant products. The major challenge is no longer our ability to produce improved vaccine products but in selecting the appropriate population to be vaccinated and in meet-

ing the enormous cost of producing vaccines to cover all serotypes and relevant subtypes. During the past several years, HBAT, the new despeciated heptavalent equine antitoxin, has become available and achieved licensure by the US FDA in 2013. Compared to previous antitoxins, HBAT is expected to be safer and less reactogenic, and an adequate supply will be available in the SNS to meet all contingencies. Next-generation antitoxins derived from mixtures of mAbs are in development and represent a stable source with a superior biological half-life.

Efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Early drug discovery efforts were hampered by the lack of information on targets and the absence of the structural information on BoNT. Current research has been aided enormously by the availability of precise structural information and by knowledge of the mechanism of LC-mediated proteolysis of SNARE proteins [42, 60, 136]. Results to date indicate that a number of SMI and peptide inhibitors are effective in inhibiting BoNT LC-mediated protease activity in cell-free in vitro systems [38, 39, 49, 89, 184, 246].

Development of safe and effective metalloprotease inhibitors with in vivo efficacy will no doubt be difficult, but the data with new quinolinol compounds are encouraging [234]. Some of the challenges involve targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartment and increasing the persistence of the drugs to match the duration of the toxin [89, 108, 276]. In addition, different inhibitors may be needed for each serotype, requiring multiple parallel efforts. A more complete characterization of BoNT receptors and a better understanding of the internalization process have recently become available and will aid in accomplishing these objectives by refining drug delivery methodologies [58, 123].

It may also be necessary to accelerate the removal of truncated SNARE proteins from the nerve terminal, to introduce noncleavable SNARE analogs for a more rapid recovery [178] and to accelerate degradation of BoNT/A LC by the ubiquitin–proteosome system [255] ([137], see also Chap. 9 this volume). The latter may be especially relevant for treatment of intoxication by persistent serotypes such as BoNT/A and BoNT/B [10, 97, 128, 129].

Other approaches such as combinations of K^+ channel blockers and Ca^{2+} channel activators appear to be promising for eliciting rapid reversal of paralysis following intoxication by BoNT/A [13]. Finally, growth factors such as IGF-1 can increase the rate of recovery and their use would be particularly useful following intoxication by the more persistent serotypes. Progress made during the last decade suggests that pharmacological treatments for BoNT intoxication may soon be a reality.

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The experimental protocols were approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense

and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

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References

1. Adler M (2006) Inhalation toxicology of botulinum neurotoxin. In: Salem H, Katz SA (eds) Inhalation toxicology, 2nd edn. Taylor & Francis, Boca Raton, pp 963–972
2. Adler M, Nicholson JD (2008) Evaluation of toosendanin as a botulinum neurotoxin antagonist. Botulinum J 1:208–218
3. Adler M, Oliveira AC, Albuquerque EX, Mansour NA, Eldefrawi AT (1979) Reaction of tetraethylammonium with the open and closed conformation of the acetylcholine receptor ionic channel complex. J Gen Physiol 74:129–152
4. Adler M, Scovill J, Parker G, Lebeda FJ, Piotrowski J, Deshpande SS (1995) Antagonism of botulinum toxin-induced muscle weakness by 3,4-diaminopyridine in rat phrenic nerve-hemidiaphragm preparations. Toxicon 33:527–537
5. Adler M, MacDonald DA, Sellin LC, Parker GW (1996) Effect of 3,4-diaminopyridine on rat extensor digitorum longus muscle paralyzed by local injection of botulinum neurotoxin. Toxicon 34:237–249
6. Adler M, Dinterman RE, Wannemacher RW (1997) Protection by the heavy metal chelator N, N, N', N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) against the lethal action of botulinum neurotoxin A and B. Toxicon 35:1089–1100
7. Adler M, Nicholson JD, Cornille F, Hackley BE Jr (1998) Efficacy of a novel metalloprotease inhibitor on botulinum neurotoxin B activity. FEBS Lett 429:234–238
8. Adler M, Shafer H, Hamilton T, Petrali JP (1999) Cytotoxic actions of the heavy metal chelator TPEN on NG108-15 neuroblastoma-glioma cells. NeuroToxicology 20:571–582
9. Adler M, Capacio B, Deshpande SS (2000) Antagonism of botulinum toxin A-mediated muscle paralysis by 3,4-diaminopyridine delivered via osmotic minipumps. Toxicon 38:1381–1388
10. Adler M, Keller JE, Sheridan RE, Deshpande SS (2001) Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in the rat EDL muscle. Toxicon 39:233–243
11. Adler M, Deshpande S, Mangel P, Nicholson J, Apland J (2008) Reversal of BoNT/A-mediated paralysis by 3,4-diaminopyridine and roscovitine in mouse phrenic nerve-hemidiaphragm preparations. 45th Annual Interagency Botulinum Research Coordinating Committee Meeting, Philadelphia, PA
12. Adler M, Deshpande SS, Apland JP (2010) Simultaneous or sequential administration of botulinum neurotoxin E does not reduce the duration of paralysis caused by botulinum neurotoxin A in rat EDL muscle. Botulinum J 1:442–456
13. Adler M, Deshpande SS, Apland JP, Murray B, Borrell A (2012) Reversal of BoNT/A-mediated inhibition of muscle paralysis by 3,4-diaminopyridine and roscovitine in mouse phrenic nerve-hemidiaphragm preparations. Neurochem Int 61:866–873
14. Agarwal R, Binz T, Swaminathan S (2005) Structural analysis of botulinum neurotoxin serotype F light chain: implications on substrate binding and inhibitor design. BioChemistry 44:11758–11765
15. Ahmed SA, Smith LA (2000) Light chain of botulinum neurotoxin expressed as inclusion body from a synthetic gene is catalytically and functionally active. J Protein Chem 19:475–487
16. Aisen ML, Sevilla D, Gibson G, Kutt H, Blau A, Edelstein L, Hatch J, Blass J (1995) 3,4-Diaminopyridine as a treatment for amyotrophic lateral sclerosis. J Neurol Sci 129:21–24

17. Aldoss IT, Tashi T, Ganti AK (2009) Seliciclib in malignancies. Expert Opin Investig Drugs 18:1957–1965
18. Al-Saleem FH, Ancharski DM, Joshi SG, Elias M, Singh A, Nasser Z, Simpson LL (2012) Analysis of the mechanisms that underlie absorption of botulinum toxin by the inhalation route. Infect Immun 80:4133–4142
19. Ambache N (1951) A further survey of the action of *Clostridium botulinum* toxin upon different type of autonomic nerve fibre. J Physiol (Lond) 113:1–17
20. Amersdorfer P, Wong C, Chen S, Smith T, Deshpande S, Sheridan R, Finnern R, Marks JD (1997) Molecular characterization of murine humoral immune response to botulinum neurotoxin type A binding domain as assessed by using phage antibody libraries. Infect Immun 65:3743–3752
21. Anderson PD (2012) Bioterrorism: toxins as weapons. J Pharm Pract 25:121–129
22. Aoki KR (2002) Physiology and pharmacology of therapeutic botulinum neurotoxins. Curr Probl Dermatol 30:107–116
23. Aoki KR, Smith LA, Atassi MZ (2010) Mode of action of botulinum neurotoxins: current vaccination strategies and molecular immune recognition. Crit Rev Immunol 30:167–187
24. Arndt JW, Chai Q, Christian T, Stevens RC (2006) Structure of botulinum neurotoxin type D light chain at 1.65 Å resolution: repercussions for VAMP-2 substrate specificity. BioChemistry 45:3255–3262
25. Arnon SS (1995) Botulism as an intestinal toxaemia. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL (eds) Infections of the gastrointestinal tract. Raven Press, New York, pp 257–271
26. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K (2001) Botulinum toxin as a biological weapon: medical and public health management. JAMA 285:1059–1070
27. Arnon SS, Schechter R, Maslanka SE, Jewell NP, Hatheway CL (2006) Human botulism immune globulin for the treatment of infant botulism. N Engl J Med 354:462–471
28. Atassi MZ, Dolimbek BZ (2004) Mapping of the antibody-binding regions on the HN-domain (residues 449–859) of botulinum neurotoxin A with antitoxin antibodies from four host species. Full profile of the antigenic regions of the H-chain of botulinum neurotoxin A. Protein J 23:39–52
29. Atlas RM (1998) The medical threat of biological weapons. Crit Rev Microbiol 24:157–168
30. Bakry N, Kamata Y, Simpson LL (1991) Lectins from *Triticum vulgaris* and *Limax flavus* are universal antagonists of botulinum neurotoxin and tetanus toxin. J Pharmacol Exp Ther 258:830–836
31. Baldwin MR, Tepp WH, Przedpelski A, Pier CL, Bradshaw M, Johnson EA, Barbieri JT (2008) Subunit vaccine against the seven serotypes of botulism. Infect Immun 76:1314–1318
32. Barash JR, Arnon SS (2013) A novel strain of clostridium botulinum that produces type B and type H botulinum toxins. J Infect Dis [epub ahead of print] PMID 24106296
33. Bavari S, Pless DD, Torres ER, Lebeda FJ, Olson MA (1998) Identifying the principal protective antigenic determinant of type A botulinum neurotoxin. Vaccine 16:1850–1856
34. Bhutani M, Ralph E, Sharpe MD (2005) Acute paralysis following "a bad potato": a case of botulism. Can J Anaesth 52:433–4336
35. Black RE, Gunn RA (1980) Hypersensitivity reactions associated with botulinal antitoxin. Am J Med 69:567–570
36. Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365:160–163
37. Bogdan J, Spinelli B, Gottdiener P, Harris R, McInnis J, Hollomon E, Ranallo R (2013) 10 Years of developing botulinum neurotoxin (BoNT) therapeutics at NIAID. 50th Annual Interagency Botulinum Research Coordinating Committee Meeting, Annapolis, MD
38. Boldt GE, Eubanks LM, Janda KD (2006a) Identification of a botulinum neurotoxin A protease inhibitor displaying efficacy in a cellular model. Chem Commun 29:3063–3065

39. Boldt GE, Kennedy JP, Janda KD (2006b) Identification of a potent botulinum neurotoxin A protease inhibitor using in situ lead identification chemistry. Org Lett 8:1729–1732
40. Boles J, West M, Montgomery V, Tammariello R, Pitt ML, Gibbs P, Smith L, LeClair RD (2006) Recombinant C fragment of botulinum neurotoxin B serotype (rBoNTB (HC)) immune response and protection in the rhesus monkey. Toxicon 47:877–884
41. Brashear A (2010) Botulinum toxin type A: exploring new indications. Drugs Today (Barc) 46:671–682
42. Breidenbach MA, Brunger AT (2004) Substrate recognition strategy for botulinum neurotoxin serotype A. Nature 432:925–929
43. Brin MF (2009) Basic and clinical aspects of BOTOX. Toxicon 54:676–682
44. Brooks VB (1956) An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. J Physiol (Lond) 134:264–277
45. Buraei Z, Schofield G, Elmslie KS (2007) Distinct kinetic changes in neurotransmitter release after SNARE protein cleavage. Neuropharmacology 52:883–894
46. Burgen ASV, Dickens F, Zatman LJ (1949) The action of botulinum toxin on the neuromuscular junction. J Physiol (Lond) 109:10–24
47. Burnett JC, Schmidt JJ, Stafford RG, Panchal RG, Nguyen TL, Hermone AR, Vennerstrom JL, McGrath CF, Lane DJ, Sausville EA, Zaharevitz DW, Gussio R, Bavari S (2003) Novel small molecule inhibitors of botulinum neurotoxin A metalloprotease activity. Biochem Biophys Res Commun 310:84–93
48. Burnett JC, Schmidt JJ, McGrath CF, Nguyen TL, Hermone AR, Panchal RG, Vennerstrom JL, Kodukula K, Zaharevitz DW, Gussio R, Bavari S (2005) Conformational sampling of the botulinum neurotoxin serotype A light chain: implications for inhibitor binding. Bioorg Med Chem 13:333–341
49. Burnett JC, Ruthel G, Stegmann CM, Panchal RG, Nguyen TL, Hermone AR, Stafford RG, Lane DJ, Kenny TA, McGrath CF, Wipf P, Stahl AM, Schmidt JJ, Gussio R, Brunger AT, Bavari S (2007a) Inhibition of metalloprotease botulinum serotype A from a pseudo-peptide binding mode to a small molecule that is active in primary neurons. J Biol Chem 282:5004–5501
50. Burnett JC, Opsenica D, Sriraghavan K, Panchal RG, Ruthel G, Hermone AR, Nguyen TL, Kenny TA, Lane DJ, McGrath CF, Schmidt JJ, Vennerstrom JL, Gussio R, Solaja BA, Bavari S (2007b) A refined pharmacophore identifies potent 4-amino-7-chloroquinoline-based inhibitors of the botulinum neurotoxin serotype A metalloprotease. J Med Chem 50:2127–2136
51. Burnett JC, Wang C, Nuss JE, Nguyen TL, Hermone AR, Schmidt JJ, Gussio R, Wipf P, Bavari S (2009) Pharmacophore-guided lead optimization: the rational design of a non-zinc coordinating, sub-micromolar inhibitor of the botulinum neurotoxin serotype A metalloprotease. Bioorg Med Chem Lett 19:5811–5813
52. Byrne MP, Smith TJ, Montgomery VA, Smith LA (1998) Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate. Infect Immun 66:4817–4822
53. Byrne MP, Titball RW, Holley J, Smith LA (2000) Fermentation, purification and efficacy of a recombinant vaccine candidate against botulinum neurotoxin serotype F from *Pichia pastoris*. Protein Expr Purif 18:327–337
54. Čapková K, Hixon MS, McAllister LA, Janda KD (2008) Toward the discovery of potent inhibitors of botulinum neurotoxin A: development of a robust LC MS based assay operational from low to subnanomolar enzyme concentrations. Chem Commun (Camb) 14(30):3525–3527
55. Carruthers J, Carruthers A (2009) Botulinum toxin in facial rejuvenation: an update. Dermatol Clin 27:417–425
56. CDC (2010) Investigational heptavalent botulinum antitoxin (HBAT) to replace licensed botulinum antitoxin AB and investigational botulinum antitoxin E. MMWR 59:299
57. CDC (2011) Notice of CDC's discontinuation of investigational pentavalent (ABCDE) botulinum toxoids vaccine for workers at risk of occupational exposure to botulinum toxins. MMWR 60:1454–1455

58. Chai Q, Arndt JW, Dong M, Tepp WH, Johnson EA, Chapman ER, Stevens RC (2006) Structural basis of cell surface receptor recognition by botulinum neurotoxin B. Nature 444:1090–1100
59. Charles PD (2004) Botulinum neurotoxin serotype A: a clinical update on non-cosmetic uses. Am J Health Syst Pharm 61:S11–S23
60. Chen S, Barbieri JT (2006) Unique substrate recognition by botulinum neurotoxins serotypes A and E. J Biol Chem 281:10906–10911
61. Cheng LW, Stanker LH, Henderson IITD, Lou J, Marks JD (2009) Antibody protection against botulinum neurotoxin intoxication in mice. Infect Immun 77:4305–4313
62. Cherington M (1998) Clinical spectrum of botulism. Muscle Nerve 21:701–710
63. Cherington M, Ryan DW (1968) Botulism and guanidine. N Engl J Med 278:931–933
64. Cherington M, Schultz D (1977) Effect of guanidine, germine and steroids in a case of botulism. Clin Toxicol 11:19–25
65. Chertow DS, Tan ET, Maslanka S, Schulte J, Bresnitz EA, Weisman RS, Bernstein J, Marcus SM, Kumar S, Malecki J, Sobel J, Braden CR (2006) Botulism in 4 adults following cosmetic injections with an unlicensed, highly concentrated botulinum preparation. JAMA 296:2476–2479
66. Clapp B, Golden S, Maddaloni M, Staats HF, Pascual DW (2010) Adenovirus F protein as a delivery vehicle for botulinum B. BMC Immunol 11:36
67. Clayton MA, Clayton JM, Brown DR, Middlebrook JL (1995) Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli*. Infect Immun 63:2738–2742
68. Cohen FL, Hardin SB, Nehring W, Keough MA, Laurenti S, McNabb J, Platis C, Weber C (1988) Physical and psychosocial health status 3 years after catastrophic illness-botulism. Issues Ment Health Nurs 9:387–398
69. Critchley EM, Hayes PJ, Isaacs PE (1989) Outbreak of botulism in north west England and Wales, June 1989. Lancet 2:849–853
70. Cull-Candy SG, Lundh H, Thesleff S (1976) Effects of botulinum toxin on neuromuscular transmission in the rat. J Physiol (Lond) 260:177–203
71. Davis LE, Johnson JK, Bicknell JM, Levy H, McEvoy KM (1992) Human type A botulism and treatment with 3,4-diaminopyridine. Electromyogr Clin Neurophysiol 32:379–383
72. Delgado-Martínez I, Nehring RB, Sørensen JB (2007) Differential abilities of SNAP-25 homologs to support neuronal function. J Neurosci 27:9380–9391
73. Dembek ZF, Smith LA, Rusnak JM (2007) Botulism: cause, effects, diagnosis, clinical and laboratory identification, and treatment modalities. Disaster Med Public Health Prep 1:122–134
74. Deshpande SS, Sheridan RE, Adler M (1995) A study of zinc-dependent metalloendopeptidase inhibitors as pharmacological antagonists in botulinum neurotoxin poisoning. Toxicon 33:551–557
75. Deshpande SS, Sheridan RE, Adler M (1997) Efficacy of certain quinolines as pharmacological antagonists in botulinum neurotoxin poisoning. Toxicon 35:433–445
76. DeStefino NR, Pilato AA, Dittrich M, Cherry SV, Cho S, Stiles JR, Meriney SD (2010) (R)-roscovitine prolongs the mean open time of unitary N-type calcium channel currents. Neuroscience 167:838–849
77. Devers KG, Nine JS (2010) Autopsy findings in botulinum toxin poisoning. J Forensic Sci 55:1649–1651
78. Dickerson TJ, Janda KD (2006) The use of small molecules to investigate molecular mechanisms and therapeutic targets for treatment of botulinum neurotoxin A intoxication. ACS Chem Biol 1:359–369
79. Dong M, Richards DA, Goodnough MC, Tepp WH, Johnson EA, Chapman ER (2003) Synaptotagmins I and II mediate entry of botulinum neurotoxin into cells. J Cell Biol 162:1293–1303
80. Dong M, Yeh F, Tepp WH, Dean C, Johnson EA, Janz R, Chapman ER (2006) SV2 is the protein receptor for botulinum neurotoxin A. Science 312:592–596

81. Dong J, Thompson AA, Fan Y, Lou J, Conrad F, Ho M, Pires-Alves M, Wilson BA, Stevens RC, Marks JD (2010) A single-domain llama antibody potently inhibits the enzymatic activity of botulinum neurotoxin by binding to the non-catalytic alpha-exosite binding region. Mol Biol 397:1106–1118
82. Dressler D (2010) Comparing Botox and Xeomin for axillary hyperhidrosis. J Neural Transm 117:317–319
83. Dressler D, Eleopra R (2006) Clinical use of non-A botulinum toxins: botulinum toxin type B. Neurotox Res 9:121–125
84. Dressler D, Müchau A, Bhatia KP, Quinn NP, Bigalke H (2002) Antibody-induced botulinum toxin therapy failure: can it be overcome by increased botulinum toxin doses? Eur Neurol 47:118–121
85. Dutton JJ, Fowler AM (2007) Botulinum toxin in ophthalmology. Surv Ophthalmol 52:13–31
86. Edmunds CW, KeiperGF Jr (1924) Further studies on the action of botulinus toxin. JAMA 83:495–501
87. Eisel U, Jarausch W, Goretzki K, Henschen A, Engels J, Weller U, Hudel M, Habermann E, Niemann H (1986) Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins. EMBO J 5:2495–2502
88. Emanuel P, O'Brien T, Burans J, DasGupta BR, Valdes JJ, Eldefrawi M (1996) Directing antigen specificity towards botulinum neurotoxin with combinatorial phage display libraries. J Immunol Methods 193:189–197
89. Eubanks LM, Hixon MS, Jin W, Hong S, Clancy CM, Tepp WH, Baldwin MR, Malizio CJ, Goodnough MC, Barbieri JT, Johnson EA, Boger DL, Dickerson TJ, Janda K (2007) An *in vitro* and *in vivo* disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. Proc Natl Acad Sci U S A 104:2602–2607
90. Eubanks LM, Silhár P, Salzameda NT, Zakhari JS, Xiaochuan F, Barbieri JT, Shoemaker CB, Hixon MS, Janda KD (2010) Identification of a natural product antagonist against the botulinum neurotoxin light chain protease. ACS Med Chem Lett 1:268–272
91. Fagan RP, Neil KP, Sasich R, Luquez C, Asaad H, Maslanka S, Khalil W (2011) Initial recovery and rebound of type F intestinal colonization botulism after administration of investigational heptavalent botulinum antitoxin. Clin Infect Dis 53:e125–128
92. Fairweather NF, Lyness VA, Maskell DJ (1987) Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli*. Infect Immun 55:2541–2545
93. Fernandez-Salas E, Steward LE, Ho H, Garay PE, Sun SW, Gilmore MA, Ordas JV, Wang J, Francis J, Aoki KR (2004) Plasma membrane localization signals in the light chain of botulinum neurotoxin. Proc Natl Acad Sci U S A 101:3208–3213
94. Fiock MA, Cardella MA, Gearinger NF (1963) Studies on immunity to toxins of *Clostridium botulinum*. IX. Immunologic response of man to purified petavalent ABCDE botulinum toxoid. J Immunol 90:697–702
95. Fischer A, Montal M (2006) Characterization of clostridial botulinum neurotoxin channels in neuroblastoma cells. Neurotox Res 9:93–100
96. Fischer A, Nakai Y, Eubanks LM, Clancy CM, Tepp WH, Pellett S, Dickerson TJ, Johnson EA, Janda KD, Montal M (2009) Bimodal modulation of the botulinum neurotoxin protein-conducting channel. Proc Natl Acad Sci U S A 106:1330–1335
97. Foran PG, Mohammed N, Lisk GO, Nagwaney S, Lawrence GW, Johnson E, Smith L, Aoki KR, Dolly JO (2003) Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. J Biol Chem 278:1363–1371
98. Foster KA (2005) A new wrinkle on pain relief: re-engineering clostridial neurotoxins for analgesics. Drug Discov Today 10:563–569
99. Foster KA, Adams EJ, Durose L, Cruttwell CJ, Marks E, Shone CC, Chaddock JA, Cox CL, Heaton C, Sutton JM, Wayne J, Alexander FC, Rogers DF (2006) Re-engineering the target specificity of clostridial neurotoxins- a route to novel therapeutics. Neurotox Res 9:101–107
100. Franz DR, Pitt LM, Clayton MA, Hanes MA, Rose KJ (1993) Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In: DasGupta BR (ed)

Botulinum and tetanus neurotoxins and biomedical aspects. Plenum Press, New York, pp 473–476
101. Franz DR, Jahrling PB, Friedlander AM, McClain DJ, Hoover DL, Bryne WR, Pavlin JA, Christopher GW, Eitzen EM Jr (1997) Clinical recognition and management of patients exposed to biological warfare agents. JAMA 278:399–411
102. Fujihashi K, Staats HF, Kozaki S, Pascual DW (2007) Mucosal vaccine development for botulinum intoxication. Expert Rev Vaccines 6:34–45
103. Gelzleichter TR, Myers MA, Menton RG, Niemuth NA, Matthews MC, Langford MJ (1999) Protection against botulinum toxins provided by passive immunization with botulinum human immune globulin: evaluation using an inhalation model. J Appl Toxicol 19:S35–S38
104. Gill DM (1982) Bacterial toxins: a table of lethal amounts. Microbiol Rev 46:86–94
105. Giménez DF, Ciccarelli AS (1970) Another type of *Clostridium botulinum*. Zentralbl Bakteriol Orig 215:221–224
106. Goldman ER, Anderson GP, Bernstein RW, Swain MD (2010) Amplification of immunoassays using phage-displayed single domain antibodies. J Immunol Methods 352:182–185
107. Gomez S, Queiroz LS (1982) The effects of black widow spider venom on the innervation of muscles paralysed by botulinum toxin. Q J Exp Physiol 67:495-506
108. Goodnough MC, Oyler G, Fishman PS, Johnson EA, Neale EA, Keller JE, Tepp WH, Clark M, Hartz S, Adler M (2002) Development of a delivery vehicle for intracellular transport of botulinum neurotoxin antagonists. FEBS Lett 513:163–168
109. Gu S, Rumpel S, Zhou J, Strotmeier J, Bigalke H, Perry K, Shoemaker CB, Rummel A, Jin R (2012) Botulinum neurotoxin is shielded by NTNHA in an interlocked complex. Science 335:977–981
110. Guendel I, Agbottah ET, Kehn-Hall K, Kashanchi F (2010) Inhibition of human immunodeficiency virus type-1 by cdk inhibitors. Aids Res Ther 7:1–14
111. Gul N, Smith LA, Ahmed SA (2010) Light chain separated from the rest of the type a botulinum neurotoxin molecule is the most catalytically active form. PloS One 5(9):e12872
112. Guyton AC, MacDonald MA (1947) Physiology of botulinum toxin. Arch Neurol Psych 57:578-592
113. Hart MK, Saviolakis GA, Welkos SL, House RV (2012) Advanced development of the rF1V and rBV A/B vaccines. Adv Prev Med 2012:731604
114. Hatheway CL (1990) Toxigenic clostridia. Clin Microbiol Rev 3:66–98
115. Hatheway CL, Snyder JD, Seals JE, Edell TA, Lewis GE Jr (1984) Antitoxin levels in botulism patients treated with trivalent equine botulism antitoxin to toxin types A, B, and E. J Infect Dis 150:407–412
116. Henderson DA (1999) The looming threat of bioterrorism. Science 283:1279–1282
117. Hermone AR, Burnett JC, Nuss JE, Tressler LE, Nguyen TL, Solaja BA, Vennerstrom JL, Schmidt JJ, Wipf P, Bavari S, Gussio R (2008) Three-dimensional database mining identifies a unique chemotype that unites structurally diverse botulinum neurotoxin serotype A inhibitors in a three-zone pharmacophore. ChemMedChem 3:1905–1912
118. Hewlett RT (1929) Bacillus botulinus. A system of bacteriology in relation to medicine, vol III. Med Research Council, London, pp 373–406
119. Hibbs RG, Weber JT, Corwin A, Allos BM, Abd ERehimMS, El Sharkawy S, Sarn JE, McKee KT Jr (1996) Experience with the use of an investigational $F(ab')_2$ heptavalent botulism immune globulin of equine origin during an outbreak of type E botulism in Egypt. Clin Infect Dis 23:337–340
120. Hill SE, Iqbal R, Cadiz CL, Le J (2013) Foodborn botulism treated with heptavalent botulism antitoxin. Ann Pharmacother 47:e12
121. Ho CY, Strobel E, Ralbovsky J, Galemmo RA Jr (2005) Improved solution-and solid-phase preparation of hydroxamic acids from esters. J Org Chem 70:4873–4875
122. Howe JR, Ritchie JM (1991) On the active form of 4-aminopyridine: block of K currents in rabbit Schwann cells. J Physiol (Lond) 433:183–205
123. Jin R, Rummel A, Binz T, Brunger AT (2006) Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. Nature 444:1092–1095

124. Johnson SK, Zhang W, Smith LA, Hywood-Potter KJ, Todd Swanson S, Schlegel VL, Meagher MM (2003) Scale-up of the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in Pichia pastoris. Protein Expr Purif 32:1–9
125. Jurasinski CV, Leith E, Dang Do AN, Schengrund CL (2001) Correlation of cleavage of SNAP-25 with muscle function in a rat model of botulinum neurotoxin type A induced paralysis. Toxicon 39:1309–1315
126. Kao I, Drachman DB, Price DL (1976) Botulinum toxin: mechanism of presynaptic blockade. Science 193:1256–1258
127. Keller JE (2008) Characterization of new formalin-detoxified botulinum neurotoxin toxoids. Clin Vaccine Immunol 15:1374–1379
128. Keller JE, Neale EA (2001) The role of the synaptic protein SNAP-25 in the potency of botulinum neurotoxin A. J Biol Chem 276:13476–13482
129. Keller JE, Neale EA, Oyler G, Adler M (1999) Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett 456:137–142
130. Kimura K, Fujii N, Tsuzuki K, Murakami T, Indoh T, Yokosawa N, Oguma K (1991) Cloning of the structural gene for *Clostridium botulinum* type C1 toxin and whole nucleotide sequence of its light chain component. Appl Environ Microbiol 57:1168–1172
131. Knockaert M, Greengard P, Meijer L (2002) Pharmacologic inhibitors of cycline dependent kinase. Trends Pharmacol Sci 23:417–425
132. Kobayashi H, Fujisawa K, Saito Y, Kamijo M, Oshima S, Kubo M, Eto Y, Monma C, Kitamura M (2003) A botulism case of a 12-year old girl caused by intestinal colonization of *Clostridium botulinum* type Ab. Jpn J Infect Dis 56:73–74
133. Kobayashi R, Kohda T, Kataoka K, Ihara H, Kozaki S, Pascual DW, Staats HF, Kiyono H, McGhee JR, Fujihashi K (2005) A novel neurotoxoid vaccine prevents mucosal botulism. J Immunol 174:2190–2195
134. Kohda T, Ihara H, Seto Y, Tsutsuki H (2007) Differential contribution of the residues in C-terminal half of the heavy chain of botulinum neurotoxin type B to its binding to the ganglioside GT1b and the synaptotagmin 2/GT1b complex. Microb Pathog 42:72–79
135. Koriazova LK, Montal M (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat Struct Biol 10:13–18
136. Kumaran D, Rawat R, Ludivico ML, Ahmed SA, Swaminathan S (2008) Structure-and substrate-based inhibitor design for *Clostridium botulinum* neurotoxin serotype A. J Biol Chem 283:18883–18891
137. Kuo CL, Oyler GA, Shoemaker CB (2011) Accelerated neuronal cell recovery from botulinum neurotoxin intoxication by targeted ubiquitination. PloS One 6(5):e20352
138. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat Struct Biol 5:898–902
139. Lebeda FJ, Adler M (2010) Hydrodynamic models for the diffusivity of type A botulinum neurotoxin. Botulinum J 1:393–406
140. Lebeda FJ, Cer RZ, Stephens RM, Mudunuri U (2010) Temporal characteristics of botulinum neurotoxin therapy. Expert Rev Neurother 10:93–103
141. Lee JS, Pushko P, Parker MD, Dertzbaugh MT, Smith LA, Smith JF (2001) Candidate vaccine against botulinum neurotoxin serotype A derived from a Venezuelan equine encephalitis virus vector system. Infect Immun 69:5709–5715
142. Le Tourneau C, Faivre S, Laurence V, Delbaldo C, Vera K, Girre V, Chiao J, Armour S, Frame S, Green SR, Gianella-Borradori A, Diéras V, Raymond E (2010) Phase I evaluation of seliciclib (R-roscovitine), a novel oral cyclin-dependent kinase inhibitor, in patients with advanced malignancies. Eur J Cancer 46:3243–3250
143. Li MF, Shi YL (2006) Toosendanin interferes with pore formation of botulinum toxin type A in PC12 cell membrane. Acta Pharmacol Sin 27:66–70
144. Li B, Peet NP, Butler MM, Burnett JC, Moir DT, Bowlin TL (2010) Small molecule inhibitors as countermeasures for botulinum neurotoxin intoxication. Molecules 16:202–220

145. Lin MJ, Lin-Shiau SY (1997) Multiple types of Ca2+channels in mouse motor nerve terminals. Eur J Neurosci 9:817–823
146. Lindström M, Korkeala H (2006) Laboratory diagnostics of botulism. Clin Microbiol Rev 19:298–314
147. Liu Z, Zhang C, Li Y, Song C, Sun Y, Wei Y, Xu Z, Yang A, Xu Z, Yang K, Jin B (2012) High sensitivity ELISA for detection of botulinum neurotoxin serotype F. Hybridoma (Larchmt) 31:233–239
148. Lundh H, Leander S, Thesleff S (1977) Antagonism of the paralysis produced by botulinum toxin in the rat. The effects of tetraethylammonium, guanidine and 4-aminopyridine. J Neurol Sci 32:29–43
149. Maddaloni M, Staats HF, Mierzejewska D, Hoyt T, Robinson A, Callis G, Kozaki S, Kiyono H, McGhee JR, Fujihashi K, Pascual DW (2006) Mucosal vaccine targeting improves onset of mucosal and systemic immunity to botulinum neurotoxin A. J Immunol 177:5524–5532
150. Mann J (1983) Prolonged recovery from type A botulism. N Engl J Med 309:1522–1523
151. Marcus SM (2009) Reflections on the care of a patient severely poisoned by 'rogue' botulinum toxin and rendered paralysed for a protracted hospital stay. The Botulinum J 1:318–339
152. Marks JD (2004) Deciphering antibody properties that lead to potent botulinum neurotoxin neutralization. Movement Disorders Supplement 8:S101–S108
153. Matarasso A, Shafer D (2009) Botulinum neurotoxin type A-ABO (Dysport): clinical indications and practice guide. Aesthet Surg J 29:S72–S79
154. Mayers CN, Holley JL, Brooks T (2001) Antitoxin therapy for botulinum intoxication. Rev Med Microbiol 12:29–37
155. Mayorov AV, Willis B, Di Mola A, Adler D, Borgia J, Jackson O, Wang J, Luo Y, Tang L, Knapp RJ, Natarajan C, Goodnough MC33, Zilberberg N, Simpson LL, Janda KD (2010) Symptomatic relief of botulinum neurotoxin/A intoxication with aminopyridines: a new twist on an old molecule. ACS Chem Biol 5:1183–1191
156. McClue SJ, Stuart I (2008) Metabolism of the trisubstituted purine cyclin-dependent kinase inhibitor seliciclib (R-roscovitine) in vitro and in vivo. Drug Metab Dispos 36:561–570
157. Meng J, Ovsepian SV, Wang J, Pickering M, Sasse A, Aoki KR, Lawrence GW, Dolly JO (2009) Activation of TRPV1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with antinociceptive potential. J Neurosci 29:4981–4992
158. Merino I, Thompson JD, Millard CB, Schmidt JJ, Pang YP (2006) Bis-imidazoles as molecular probes for peripheral sites of the zinc endopeptidase of botulinum neurotoxin serotype A. Bioorg Med Chem 14:3583–3591
159. Merz B, Bigalke H, Stoll G, Naumann M (2003) Botulism type B presenting as pure autonomic dysfunction. Clin Auton Res 13:337–338
160. Mesngon M, McNutt P (2011) Alpha-latrotoxin rescues SNAP-25 from BoNT/A-mediated proteolysis in embryonic stem cell-derived neurons. Toxins 3:489–503
161. Meyer K, Ng H, Parman T, D'Andrea A, Harrison T, Green C, Ma J, Cao L, Shimizu B, Der K, Mirsalis J (2011) Nonclinical safety evaluation of XOMA 3AB, a novel triple monoclonal antibody drug product targeting botulinum toxin type A, in Sprague-Dawley rats. Poster presented at the Public Health Emergency Medical Countermeasures Enterprise (PHEMCE) Meeting, January 10–12, 2011, Washington, DC
162. Middlebrook JL, Franz DR (1997) Botulinum toxins. In: Zajtchuk R, Bellamy RF (eds) Textbook of military medicine: medical aspects of chemical and biological warfare, part I. Borden Institute, Washington, DC, pp 643–654
163. Moe ST, Thompson AB, Smith GM, Fredenburg RA, Stein RL, Jacobson AR (2009) Botulinum neurotoxin serotype A inhibitors: small-molecule mercaptoacetamide analogs. Bioorg Med Chem 17:3072–3079
164. Molgó J, Lundh H, Thesleff S (1980) Potency of 3,4-diaminopyridine and 4-aminopyridine on mammalian neuromuscular transmission and the effect of pH changes. Eur J Pharmacol 61:25–34

165. Molgó J, Lemeignan M, Thesleff S (1987) Aminoglycosides and 3,4-diaminopyridine on neuromuscular block cause by botulinum type A toxin. Muscle Nerve 10:464–470
166. Moller V, Scheibel I (1960) Preliminary report on the isolation of an apparently new type of *Clostridium botulinum*. Acta Pathol Microbiol Scand 48:80
167. Montecucco C (1986) How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem Sci 11:314–317
168. Montecucco C, Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem Sci 18:324–327
169. Montecucco C, Papini E, Schiavo G (1994) Bacterial protein toxins penetrate cells via a four-step mechanism. FEBS Lett 346:92–98
170. Muñoz-Caro C, Niño A (2002) The nature of the receptor site for the reversible K^+channel blocking by aminopyridines. Biophys Chem 96:1–14
171. Mustafa W, Al-Saleem FH, Nasser Z, Olson RM, Mattis JA, Simpson LL, Schnell MJ (2011) Immunization of mice with the non-toxic HC50 domain of botulinum neurotoxin presented by rabies virus particles induces a strong immune response affording protection against high-dose botulinum neurotoxin challenge. Vaccine 29:4638–4645
172. Nakamura Y, Takahashi T (2007) Developmental changes in potassium currents at the rat calyx of held presynaptic terminal. J Physiol (Lond) 581:1101–1112
173. Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, Sato K, Sekiguchi M, Takahashi M, Kozaki S (1996) The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1. FEBS Lett 378:253–257
174. Nowakowski A, Wang C, Powers DB, Armersdorfer P, Smith TJ, Montgomery VA, Sheridan R, Blake R, Smith LA, Marks JD (2002) Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. Proc Natl Acad Sci U S A 99:11346–11135
175. Nuss JE, Dong Y, Wanner LM, Ruthel G, Wipf P, Gussio R, Vennerstrom JL, Bavari S, Burnett JC (2010) Pharmacophore refinement guides the rational design of nanomolar-range inhibitors of the botulinum neurotoxin serotype A metalloprotease. ACS Med Chem Lett 14:301–305
176. Oost T, Sukonpan C, Brewer M, Goodnough M, Tepp W, Johnson EA, Rich DH (2003) Design and synthesis of substrate-based inhibitors of botulinum neurotoxin type B metalloprotease. Biopolymers 71:602–619
177. Opsenica I, Burnett JC, Gussio R, Opsenica D, Todorović N, Lanteri CA, Sciotti RJ, Gettayacamin M, Basilico N, Taramelli D, Nuss JE, Wanner L, Panchal RG, Solaja BA, Bavari S (2011) A chemotype that inhibits three unrelated pathogenic targets: the botulinum neurotoxin serotype A light chain, P. falciparum malaria, and the Ebola filovirus. J Med Chem 54:1157–1169
178. O'Sullivan GA, Mohammed N, Foran PG, Lawrence GW, Dolly JO (1999) Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage resistant SNAP-25. Identification of the minimal essential C-terminal residues. J Biol Chem 274:36897–36904
179. Pang YP (2001) Successful molecular dynamics simulation of two zinc complexes bridged by a hydroxide in hosphotriesterase using the cationic dummy atom method. Proteins 45:183–189
180. Pang YP, Vummenthala A, Mishra RK, Park JG, Wang S, Davis J, Millard CB, Schmidt JJ (2009) Potent new small-molecule inhibitor of botulinum neurotoxin serotype A endopeptidases developed by synthesis-based computer-aided molecular design. PloS One 4(11):e7730
181. Pang YP, Davis J, Wang S, Park JG, Nambiar MP, Schmidt JJ, Millard CB (2010) Small molecules showing significant protection of mice against botulinum neurotoxin serotype A. PloS One 5(4):e10129
182. Pantano S, Montecucco C (2013) The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. Cell Mol Life Sci PMID: 23749048 (Epub ahead of print)
183. Park J-B, Simpson LL (2003) Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. Infect Immun 71:1147–1154

184. Park JG, Sill PC, Makiyi EF, Garcia-Sosa AT, Millard CB, Schmidt JJ, Pang YP (2006) Serotype-selective, small-molecule inhibitors of the zinc endopeptidase of botulinum neurotoxin serotype A. Bioorg Med Chem 14:395–408
185. Penner R, Dreyer F (1986) Two different presynaptic calcium currents in mouse motor nerve terminals. Pflugers Arch 406:190–196
186. Pier CL, Tepp WH, Bradshaw M, Johnson EA, Barbieri JT, Baldwin MR (2008) Recombinant holotoxoid vaccine against botulism. Infect Immun 76:437–442
187. Pires-Alves M, Ho M, Aberle KK, Janda KD, Wilson BA (2009) Tandem fluorescent proteins as enhanced FRET-based substrates for botulinum neurotoxin activity. Toxicon 53:392–399
188. Potian JG, Thyagarajan B, Hognason K, Lebeda F, Schmidt JJ, McArdle JJ (2010) Investigation of "CRATKML" derived peptides against botulinum neurotoxin A poisoning in vivo and in vitro. Botulinum J 1:407–417
189. Potter KJ, Bevins MA, Vassilieva EV, Chiruvolu VR, Smith TJ, Smith LA et al. (1998) Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotropic yeast *Pichia pastoris*. Protein Expr Purif 13:1357–1365
190. Pozsgay M, Michaud C, Liebman M, Orlowski M (1986) Substrate and inhibitor studies of thermolysin-like neutral metalloendopeptidase from kidney membrane fractions. Comparison with bacterial thermolysin. BioChemistry 25:1292–1299
191. Ramasamy S, Liu CQ, Tran H, Gubala A, Gauci P, McAllister J, Vo T (2010) Principles of antidote pharmacology: an update on prophylaxis, post-exposure treatment recommendations and research initiatives for biological agents. Br J Pharmacol 161:721–748
192. Raphael BH, Choudoir MJ, Lúquez C, Fernández R, Maslanka SE (2010) Sequence diversity of genes encoding botulinum neurotoxin type F. Appl Environ Microbiol 76:4805–4812
193. Ravichandran E, Gong Y, Al-Saleem FH, Ancharski DM, Joshi SG, Simpson LL (2006) An initial assessment of the systemic pharmacokinetics of botulinum toxin. J Pharmacol Exp Ther 318:1343–1351
194. Ravichandran E, Al-Saleem FH, Ancharski DM, Elias MD, Singh AK, Shamim M, Gong Y, Simpson LL (2007) Trivalent vaccine against botulinum toxin serotypes A, B, and E that can be administered by the mucosal route. Infect Immun 75:3043–3054
195. Razai A, Garcia-Rodriguez C, Lou J, Geren IN, Forsyth CM, Robles Y, Tsai R, Smith TJ, Smith LA, Siegel RW, Feldhaus M, Marks JD (2005) Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. J Mol Biol 351:158–169
196. Rega PP, Bork CE, Burkholder-Allen K, Bisesi MS, Gold JP (2010) Single-breath count test: an important adjunct in the triaging of patients in a mass-casualty incident due to botulism. Prehosp Disaster Med 25:219–222
197. Rettig J, Neher E (2002) Emerging roles of presynaptic proteins in Ca^{++}-triggered exocytosis. Science 298:781–785
198. Robinson RF, Nahata MC (2003) Management of botulism. Ann Pharmacother 37:127–131
199. Rosetto O, Deloye F, Poulain B, Pellizzari R, Schiavo G, Montecucco C (1995) The metalloproteinase activity of tetanus and botulinum neurotoxins. J Physiol (Paris) 89:43–50
200. Roxas-Duncan V, Enyedy I, Montgomery VA, Eccard VS, Carrington MA, Lai H, Gul N, Yang DC, Smith LA (2009) Identification and biochemical characterization of small-molecule inhibitors of *Clostridium botulinum* neurotoxin serotype A. Antimicrob Agents Chemother 53:3478–3486
201. Ruge DR, Dunning FM, Piazza TM, Molles BE, Adler M, Zeytin FN, Tucker WC (2011) Detection of six serotypes of botulinum neurotoxin using fluorogenic reporters. Anal Biochem 411:200–209
202. Rusnak JM, Smith LA (2009) Botulinum neurotoxin vaccines: past history and recent developments. Hum Vaccin 5:794–805
203. Sakaba T, Stein A, Jahn R, Neher E (2005) Distinct kinetic changes in neurotransmitter release after SNARE protein cleavage. Science 309:491–494
204. Schiavo G, Rossetto O, Santucci A, DasGupta BR, Motecucco C (1992) Botulinum neurotoxins are zinc proteins. J Biol Chem 267:23479–23483

205. Schiavo G, Shone CC, Rosetto O, Alexander FC, Montecucco C (1993) Botulinum neurotoxin F is a zinc endopeptidase specific for VAMP/synaptobrevin. J Biol Chem 268:11516–11519
206. Schiavo G, Malizio C, Trimble WS, Polverino deLP, Milan G, Sugiyama H, Johnson EA, Montecucco C (1994) Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J Biol Chem 269:20213–20216
207. Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. Physiol Rev 80:717–766
208. Schmidt JJ, Bostian KA (1995) Proteolysis of synthetic peptides by type A botulinum neurotoxin. J Protein Chem 14:703–708
209. Schmidt JJ, Stafford RG (2002) A high affinity competitive inhibitor of type A botulinum neurotoxin protease activity. FEBS Lett 532:423–426
210. Schmidt JJ, Stafford RG (2003) Fluorigenic substrates for the protease activities of botulinum neurotoxins, serotypes A, B, and F. Appl Environ Micobiol 69:297–303
211. Schmidt JJ, Stafford RG (2005) Botulinum neurotoxin serotype F: identification of substrate recognition requirements and development of inhibitors with low nanomolar affinity. BioChemistry 44:4067–4073
212. Schmidt JJ, Stafford RG, Bostian KA (1998) Type A botulinum neurotoxin proteolytic activity: development of competitive inhibitors and implication for substrate specificity at the S1′ binding site. FEBS Lett 435:61–64
213. Schmidt JJ, Stafford RG, Millard CB (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. Anal Biochem 296:130–137
214. Segelke B, Knapp M, Kadkhodayan S, Balhorn R, Rupp B (2004) Crystal structure of *Clostridium botulinum* neurotoxin protease in a product-bound state: Evidence for noncanonical zinc protease activity. Proc Natl Acad Sci U S A 101:6888–6893
215. Shapiro RL, Hatheway C, Swerdlow DL (1998) Botulism in the United States: a clinical and epidemiological review. Ann Intern Med 129:221–228
216. Sheridan RE, Deshpande SS (1995) Interaction between heavy metal chelators and botulinum neurotoxin at the neuromuscular junction. Toxicon 33:539–549
217. Sheridan RE, Deshpande SS (1998) Cytotoxicity induced by intracellular zinc chelation in rat cortical neurons. In vitro & Mol Toxicol 11:161–169
218. Shi YL, Wang ZF (2004) Cure of experimental botulism and antibotulismic effect of toosendanin. Acta Pharmacol Sin 25:839–848
219. Shoham D (2000) Iraq's biological warfare agents: a comprehensive analysis. Crit Rev Microbiol 26:179–204
220. Shone C, Agostini H, Clancy J, Gu M, Yang H-H, Chu Y, Johnson V, Makie Taal M, McGlashan J, Brehm J, Tong X (2009) Bivalent recombinant vaccine for botulinum neurotoxin types A and B based on a polypeptide comprising their effector and translocation domains that is protective against the predominant A and B subtypes. Infect Immun 77:2795–2801
221. Siegel LS (1988) Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay. J Clin Microbiol 26:2351–2356
222. Silberstein SD, Blumenfeld AM, Cady RK, Turner IM, Lipton RB, Diener HC, Aurora SK, Sirimanne M, Degryse RE, Turkel CC, Dodick DW (2013) OnabotulinumtoxinA for treatment of chronic migraine: PREEMPT 24-week pooled subgroup analysis of patients who had acute headache medication overuse at baseline. J Neurol Sci 331:48–56
223. Šilhár P, Čapková K, Salzameda NT, Barbieri JT, Hixon MS, Janda KD (2010) Botulinum neurotoxin A protease: discovery of natural product exosite inhibitors. J Am Chem Soc 132:2868–2869
224. Silvaggi NR, Wilson D, Tzipori S, Allen KN (2008) Catalytic features of the botulinum neurotoxin A light chain revealed by high resolution structure of an inhibitory peptide complex. BioChemistry 47:5736–5745
225. Simpson LL (1982) The interaction between aminoquinolines and presynaptically acting neurotoxins. J Pharmacol Exp Ther 222:43–48

226. Simpson LL (1983) Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins. J Pharmacol Exp Ther 225:546–552
227. Simpson LL (1986) A preclinical evaluation of aminopyridines as putative therapeutic agents in the treatment of botulism. Infect Immun 52:858–862
228. Simpson LL (1988) Use of pharmacologic antagonists to deduce commonalities of biologic activity among clostridial neurotoxins. J Pharmacol Exp Ther 245:867–872
229. Simpson LL (2004) Identification of the major steps in botulinum toxin action. Annu Rev Pharmacol Toxicol 44:167–193
230. Simpson LL (2007) Balancing the benefits and risks of a botulinum vaccine. Expert Rev Vaccines 6:883–886
231. Simpson LL, Coffield JA, Bakry N (1993) Chelation of zinc antagonizes the neuromuscular blocking properties of the seven serotypes of botulinum neurotoxin as well as tetanus toxin. J Pharmacol Exp Ther 267:720–727
232. Simpson LL, Maksymowych AB, Kouguchi H, DuBois G, Bora RS, Joshi S (2005) The role of exoproteases in governing intraneuronal metabolism of botulinum toxin. Protein J 24:155–165
233. Singh BR (2006) Botulinum neurotoxin structure, engineering, and novel cellular trafficking and targeting. Neurotox Res 9:73–92
234. Singh P, Singh MK, Chaudhary D, Chauhan V, Bharadwaj P, Pandey A, Upadhyay N, Dhaked RK (2012) Small-molecule quinolinol inhibitor identified provides protection against BoNT/A in mice. Plos One 7(10):e47110
235. Smith LA (1998) Development of recombinant vaccines for botulinum neurotoxin. Toxicon 36:1539–1548
236. Smith LA, Rusnak JM (2007) Botulinum neurotoxin vaccines: past, present, and future. Crit Rev Immunol 27:303–318
237. Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, Laporte SL, Tepp WH, Bradshaw M, Johnson EA, Smith LA, Marks JD (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun 73:5450–5457
238. Sobel J (2005) Botulism. Clin Infect Dis 41:1167–1173
239. Sobel J, Dill T, Kirkpatrick CL, Riek L, Luedtke P, Damrow TA (2009) Clinical recovery and circulating botulinum toxin type F in adult patient. Emerg Infect Dis 15:968–971
240. Solaja BA, Opsenica D, Smith KS, Milhous WK, Terzić N, Opsenica I, Burnett JC, Nuss J, Gussio R, Bavari S (2008) Novel 4-aminoquinolines active against chloroquine-resistant and sensitive P. falciparum strains that also inhibit botulinum serotype A. J Med Chem 51:4388–4391
241. Sonnabend O, Sonnabend W, Heinzle R, Sigrist T, Dirnhofer R, Krech U (1981) Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in humans: report of five sudden unexpected deaths. J Infect Dis 143:22–27
242. Sørensen JB, Matti U, Wei S-H, Nehring RB, Voets T, Ashery U, Binz T, Neher E, Rettig J (2002) The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. Proc. Natl Acad Sci 99:1627–1632
243. Souayah N, Karim H, Kamin SS, McArdle J, Marcus S (2006) Severe botulism after focal injection of botulinum toxin. Neurology 67:1855–1856
244. Stahl AM, Adler M, Millard CB, Gilfillan L (2009) Accelerating botulism therapeutic product development in the department of defense. Drug Dev Res 70:303–326
245. Stowe GN, Silhár P, Hixon MS, Silvaggi NR, Allen KN, Moe St, Jacobson A, Barbieri JT, Janda KD (2010) Chirality holds the key for potent inhibition of the botulinum neurotoxin serotype A protease. Org Lett 12:756–759
246. Sukonpan C, Oost T, Goodnough M, Tepp W, Johnson EA, Rich DH (2004) Synthesis of substrates and inhibitors of botulinum neurotoxin type A metalloprotease. J Pept Res 63:181–193
247. Swaminathan S (2011) Molecular structures and functional relationships in clostridial neurotoxins. FEBS J 278:4467–4485
248. Swaminathan S, Eswaramoorthy S (2000) Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat Struct Biol 7:693–699

249. Tacket CO, Shandera WX, Mann JM, Hargrett NT, Blake PA (1984) Equine antitoxin use and other factors that predict outcome in type A foodborne botulism. Am J Med 76:794–798
250. Tang J, Park JG, Millard CB, Schmidt JJ, Pang Y-P (2007) Computer-aided lead optimization: improved small-molecule inhibitor of the zinc endopeptidase of botulinum neurotoxin serotype A. PLoS One 2(8):e761
251. Thesleff S (1989) Botulinal neurotoxins as tools in studies of synaptic mechanisms. Quart J Exp Physiol 74:1003–1017
252. Thompson DE, Brehm JK, Oultram JD, Swinfield TJ, Shone CC, Atkinson T, Melling J, Minton NP (1990) The complete amino acid sequence of the clostridium botulinum type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur J Biochem 189:73–81
253. Thompson AA, Jiao GS, Kim S, Thai A, Cregar-Hernandez L, Margosiak SA, Johnson AT, Han GW, O'Malley S, Stevens RC (2011) Structural characterization of three novel hydroxamate-based zinc chelating inhibitors of the *Clostridium botulinum* serotype A neurotoxin light chain metalloprotease reveals a compact binding site resulting from 60/70 loop flexibility. BioChemistry 59:4019–4028
254. Trollet C, Pereira Y, Burgain A, Litzler E, Mezrahi M, Sequin J, Manich M, Popoff MR, Scherman D, Bigey P (2009) Generation of high-titer neutralizing antibodies against botulinum toxins A, B, and E by DNA electrotransfer. Infect Immun 77:2221–2229
255. Tsai YC, Maditz R, Kuo CL, Fishman PS, Shoemaker CB, Oyler GA, Weissman AM (2010) Targeting botulinum neurotoxin persistence by the ubiquitin-proteosome system. Proc Natl Acad Sci U S A 107:16554–16559
256. Van Heyningen WE, Miller PA (1961) The fixation of tetanus toxin by ganglioside. J Gen Microbiol 24:107–119
257. Vennerstrom JL, Ager AL Jr, Dorn A, Andersen SL, Gerena L, Ridley RG, Milhous WK (1998) Bisquinolines. 2. Antimalarial N, N-bis(7-chloroquinolin-4-yl)heteroalkanediamines. J Med Chem 41:4360–4364
258. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M (2010) Synthetic therapeutic peptides: science and market. Drug Discov Today 15:40–56
259. Vollmer T, Blight AR, Henney HR 3rd (2009) Steady-state pharmacokinetics and tolerability of orally administered famprine sustained-release 10-mg tablets in patients with multiple sclerosis: a 2-week open-label, follow-up study. Clin Ther 31:2215–2223
260. Webb RP, Smith LA (2013) What next for vaccine developmemt? Expert Rev Vaccines 12:481–492
261. Webb RP, Smith TJ, Wright PM, Montgomery VA, Meagher MM, Smith LA (2007) Protection with recombinant *Clostridium botulinum* C1 and D binding domain subunit (Hc) vaccines against C and D neurotoxins. Vaccine 25:4273–4282
262. Webb RP, Smith TJ, Wright PM, Brown J, Smith LA (2009) Production of catalytically inactive BoNT/A1 holoprotein and comparison with BoNT/A1 subunit vaccines against toxin subtypes A1 A2 A3. Vaccine 27:4490–4497
263. Wein LM, Liu Y (2005) Analyzing a bioterror attack on the food supply: the case of botulinum toxin in milk. Proc Natl Acad Sci U S A 102:9984–9989
264. Wheeler A, Smith HS (2013) Botulinum toxins: mechanisms of action, antinociception and clinical applications. Toxicology 306:124–146
265. Whelan SM, Elmore MJ, Bodsworth NJ, Atkinson T, Minton NP (1992) The complete amino acid sequence of the *Clostridium botulinum* type-E neurotoxin derived by nucleotide-sequence analysis of the encoding gene. Eur J Biochem 204:657–667
266. Wilcox PG, Andolfatto G, Fairbarn MS, Pardy RL (1989) Long-term follow-up of symptoms, pulmonary function, respiratory muscle strength, and exercise performance after botulism. Am Rev Respir Dis 139:157–163
267. Wilder-Kofie TD, Lúquez C, Adler M, Dykes JK, Coleman JD, Maslanka SE (2011) An alternative *in vivo* method to refine the mouse bioassay for botulinum toxin detection. Comp Med 61:235–242
268. Wileman T, Harding C, Stahl P (1985) Receptor-mediated endocytosis. Biochem J 232:1–14

269. Woodruff BA, Griffin PM, McCroskey LM, Smart JF, Wainwright RB, Bryant RG, Hutwagner LC, Hatheway CL (1992) Clinical and laboratory comparison of botulism from toxin type A, B, and E in the United States, 1975–1988. J Infect Dis 166:1281–1286
270. Xu Q, Pichichero ME, Simpson LL, Elias MD, Smith LA, Zeng M (2009) An adenoviral vector-based mucosal vaccine is effective in protection against botulism. Gene Ther 16:367–375
271. Yamasaki S, Baumeister A, Binz T, Blasi J, Link E, Cornille F, Roques B, Fykse EM, Sudhof TC, Jahn R (1994) Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J Biol Chem 269:12764–12772
272. Yan Z, Chi P, Bibb JA, Ryan TA, Greengard P (2002) Roscovitine: a novel regulator of P/Q-type calcium channels and transmitter release in central neurons. J Physiol (Lond) 540:761–770
273. Yiadom KP, Muhie S, Yang DC (2005) Peptide inhibitors of botulinum neurotoxin by mRNA display. Biochem Biophys Res Commun 335:1247–1253
274. Yowler BC, Kensinger RD, Schengrund CL (2002) Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. J Biol Chem 277:32815–32819
275. Zeng M, Xu Q, Elias MD, Pichichero ME, Simpson LL, Smith LA (2007) Protective immunity against botulism provided by a single dose vaccination with an adenovirus-vectored vaccine. Vaccine 25:7540–7548
276. Zhang P, Ray R, Singh BR, Adler M, Ray P (2009) An efficient drug delivery vehicle for botulism countermeasure. BMC Pharmacol 9:12–19
277. Zhang S, Wang Y, Qiu S, Dong Y, Xu Y, Jiang D, Fu X, Zhang J, He J, Jia L, Wang L, Zhang C, Sun Y, Song H (2010) Multilocus outbreak of foodborne botulism linked to contaminated sausage in Hebei province, China. Clin Infect Dis 51:322–325
278. Zuniga JE, Schmidt JJ, Fenn T, Burnett JC, Araç D, Gussio R, Stafford RG, Badie SS, Bavari S, Brunger AT (2008) A potent peptidomimetic inhibitor of botulinum neurotoxin serotype A has a very different conformation than SNAP-25 substrate. Structure 16:1588–1597
279. Zuniga JE, Hammill JT, Drory O, Nuss JE, Burnett JC, Gussio R, Wipf P, Bavari S, Brunger AT (2010) Iterative structure-based peptide-like inhibitor design against the botulinum neurotoxin serotype A. PLoS One 5(6):e11378